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(54) Title: MULTIVALENT COMPOUNDS FOR CROSS-LINKING RECEPTORS AND USES THEREOF

(57) Abstract

Synthetic cross-linking homobivalent and heterobivalent compounds have been designed and developed. These compounds are low in molecular weight, have antagonistic or agonistic activity, and induce the association between two identical or similar natural receptors (homobivalent compounds) or induce the association between two different natural receptors (heterobivalent compounds).

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(74) Representative:

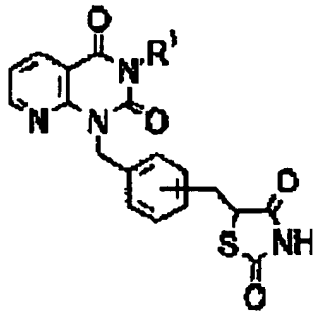
(54) PYRIDOPYRIMIDINE DERIVATIVE AND MEDICINAL COMPOSITION CONTAINING THE SAME

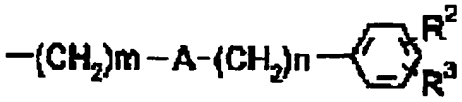
(57) Abstract:

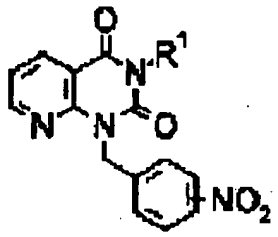
PROBLEM TO BE SOLVED: To obtain a new compound having hypoglycemic action and aldose reductase inhibitory action, and useful for treating diabetic complication, etc.

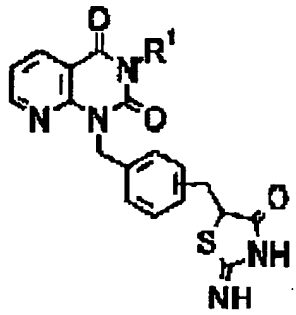
SOLUTION: This new compound is shown by formula I [R1 is a lower alkyl, of formula II (R2 and R3 are each H, a halogen, lower alkyl, etc.; (m) is 1-3; (n) is 0-1; A is O or S) or of the formula (CH2)q-B ((q) is 1-4; B is hydroxyl, a lower alkylcarbonyl, aryls, etc.)], e.g. 1-{4-[(2,4-dioxothiazolidin-5-yl) methyl] benzyl}-3-[2-(benzyloxy)ethyl]-2,4-dioxo-12,3,4-tetrahydropyrido[2,3- d] pyrimidine. The compound of formula I is obtained by the following

process: a compound of formula III is first reduced and then subjected to Meerwein arylation followed by reaction with thiourea to form a compound of formula IV, which is then hydroglyzed in the presence of an acid.









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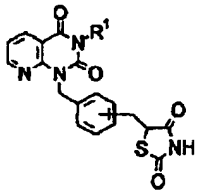
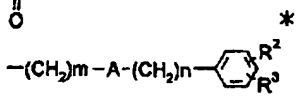
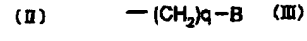
(54)【発明の名称】　ピリドピリミジン誘導体及びそれを含有する医薬組成物

(57)【要約】
【課題】血糖降下およびアルドースリダクターゼ阻害作用を併せ持ち糖尿病の各種合併症の治療に有効な薬物を得る。
【解決手段】式（I）で表されるピリドピリミジン誘導体およびそれを含有する医薬組成物。
【化1】



（I）



（II）　　 

（III）

式中、R^1、R^2はH、ハロゲン原子、低級アルキル基等、mは整数1～3、nは整数0～1を示し、AはO又はS、qは整数1～4、Bはヒドロキシ基、低級アルキルカルボニル基等を示す。

＊式中、R^1は低級アルキル基、下記式（II）または（III）である。
【化2】

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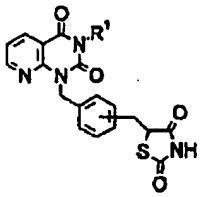
【特許請求の範囲】

【請求項１】一般式（I）で表されるピリドピリミジン誘導体。

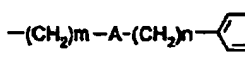
【化１】



（I）

式（I）中、R¹は低級アルキル基、あるいは下記の一般式（II）または（III）で表される基である。

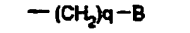
【化２】



（II）

式（II）中、R²、R³は水素原子、ハロゲン原子、低級アルキル基、低級アルコキシ基またはハロアルキル基を示し、mは１乃至３の整数を示し、nは０乃至１の整数を示し、Aは酸素原子または硫黄原子を示す。

【化３】



（III）

式（III）中、qは１乃至４の整数を示し、Bはヒドロキシ基、低級アルキルカルボニル基、置換基を有してもよいカルバモイル基、置換基を有してもよいアリール基、あるいは置換基を有してもよいアリールカルボニル基を示す。

【請求項２】請求項１に記載のピリドピリミジン誘導体を含有してなる医薬組成物。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は、新規なピリドピリミジン誘導体及びそれを含有する医薬組成物に関する。さらに詳しくは血糖降下作用およびアルドースリダクターゼ阻害作用を併せ持つ新規なピリドピリミジン誘導体、及びそれを含有する糖尿病白内障、網膜症、神経障害、腎障害などの糖尿病における各種合併症の予防および治療に有用な医薬組成物に関する。

【０００２】

【従来の技術】糖尿病治療薬としては、早くからインスリン製剤が知られ、使用されてきた。その後経口血糖降下剤として種々のビグアナイド系化合物やスルホニルウレア系化合物が開発され用いられてきた。しかしながらこれらの化合物には問題がある。すなわちインスリン製剤は現在のところ注射剤として使用されており、軽減されているとはいえ、その使用時における不便さは患者にとって大きな負担となっている。また、経口血糖降下剤には、乳酸アシドーシスや重篤な低血糖といった副作用がみられ、毒性が低く、有効な糖尿病治療薬の開発が望まれている。血糖降下剤に関連する先行技術としては、

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ＵＳＰ４５７２９１２号、ＵＳＰ４６４５６１７号およびＵＳＰ４６８７７７７号等が知られている。

【０００３】近年、糖尿病合併症の発症機序として、組織内ソルビトールの蓄積、増加が注目されグルコースなどのアルドースを還元してソルビトールに変換する酵素であるアルドースリダクターゼ（ＡＲ）の活性を阻害する化合物が、白内障、網膜症、神経障害、腎障害の治療に有用であることが文献上示唆されている［ケイ・エッチ・ギャバイ、エヌ・スパック、エス・ルーら、メタボリズム(K.H.Gabbay,N.Spack,N.Loo,et al., Metabolism), 28(Suppl.1)；471-476(1979)，デイ・ドボルーニク、エヌ・シマルドーデキュエスネ、エム・クラミら、サイエンス(D.Dvornik,N.Simard-Duquesne,M.Krami,et al., Science), 182;1146-1148(1973)，ジェイ・エッチ・キノシタ、デイ・ドボルーニク、エム・クラミら、バイオキミカ.バイオフィジカ.アクタ(J.H.Kinosita,D.Dvornik,M.Krami,et al.,Biochem.Biophys.Acta), 158;472-475(1968)参照］。これらのことよりアルドースリダクターゼ（ＡＲ）阻害剤の開発が進められている。アルドースリダクターゼ阻害剤に関連する先行技術としてはＵＳＰ４７３４４１９号、欧州特許４７１０９号、ＷＯ９２１２９７９等が知られている。しかしながら、ＡＲ阻害剤はあくまでも糖尿病合併症の対症療法剤であり、糖尿病そのものの治療効果は低いと考えられている。

【０００４】

【発明が解決しようとする課題】したがって糖尿病、加えては糖尿病合併症の根本的な治療には血糖降下剤が必要であり、さらにＡＲ阻害作用を併せ持つ血糖降下剤の開発が望まれている。

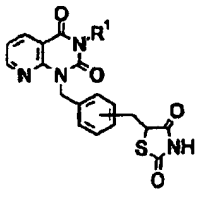
【０００５】

【課題を解決するための手段】本発明者らは、かかる状況に鑑み鋭意研究を継続した結果、新規なピリドピリミジン誘導体が該要請を満たすものであることを見いだし、以下の本発明を完成するに至った。

【０００６】（１）本発明は、一般式（I）で表されるピリドピリミジン誘導体、およびその金属塩、付加塩、水和物、溶媒和物、金属塩の水和物、金属塩の溶媒和物、付加塩の水和物、付加塩の溶媒和物である。

【０００７】

【化４】

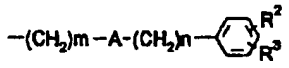


（I）

【０００８】式（I）中、R¹は低級アルキル基、あるいは下記の一般式（II）または（III）で表される基である。

【０００９】

【化5】

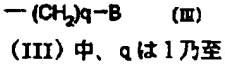


(II)

【0010】式（II）中、R^2、R^3は水素原子、ハロゲン原子、低級アルキル基、低級アルコキシ基またはハロアルキル基を示し、mは1乃至3の整数を示し、nは0乃至1の整数を示し、Aは酸素原子または硫黄原子を示す。

【0011】

【化6】



(III)

【0012】式（III）中、qは1乃至4の整数を示し、Bはヒドロキシ基、低級アルキルカルボニル基、置換基を有してもよいカルバモイル基、置換基を有してもよいアリール基、あるいは置換基を有してもよいアリールカルボニル基を示す。

【0013】（2）また本発明は、請求項1に記載のピリドピリミジン誘導体、およびその金属塩、付加塩、水和物、溶媒和物、金属塩の水和物、金属塩の溶媒和物、付加塩の水和物、付加塩の溶媒和物を含有してなる医薬組成物である。

【0014】本発明の一般式（I）のピリドピリミジン誘導体において、R^1が示す低級アルキル基は、炭素数1乃至8の直鎖、分岐鎖または環状のアルキル基が望ましく、例えばメチル、エチル、プロピル、イソプロピル、ブチル、イソブチル、sec-ブチル、ter-ブチル、ペンチル、イソペンチル、ネオペンチル、ヘキシル、オクチル等が挙げられる。また一般式（II）においてR^2、R^3が示すハロゲン原子としては、例えばフッ素原子、塩素原子、臭素原子等が挙げられ、低級アルコキシ基としては、上記の低級アルキル基と酸素原子が結合したものが挙げられる。ハロアルキル基は例えばクロロメチル、2,2－ジクロロエチル、トリフルオロメチル等が挙げられる。一般式（III）においてBが示す低級アルキルカルボニル基は例えばアセチル、プロピオニル、ブチリル、イソブチリル等が挙げられ、置換基を有していてもよいカルバモイル基は、例えばメチルカルバモイル、エチルカルバモイル、イソプロピルカルバモイル、ジメチルカルバモイル、ジエチルカルバモイル、シクロヘキシルカルバモイル、フェニルカルバモイル等が挙げられる。また置換基を有していてもよいアリール基は、例えばフェニル、ビフェニル、4－クロロフェニル、3,4－ジメトキシフェニル等が挙げられ、置換基を有していてもよいアリールカルボニル基は、例えばベンゾイル、トルオイル、アニソイル等が挙げられる。

【0015】一般式（I）で表わされるピリドピリミジン誘導体の好適な塩類としては、例えばリチウム、ナトリウム、カリウム等のアルカリ金属との塩、カルシウム、マグネシウム等のアルカリ土類金属との塩、また例

えば、塩酸、硫酸、硝酸、リン酸等の無機酸、ギ酸、酢酸、クエン酸、乳酸等の有機酸類との付加塩が挙げられる。

【0016】

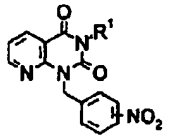
【発明の実施の形態】一般式（I）で表される本発明のピリドピリミジン誘導体の製造法を以下詳細に説明する。

（製造法）一般式（I）において、R^1は低級アルキル基、あるいは一般式（II）（式（II）中、R^2、R^3は水素原子、ハロゲン原子、低級アルキル基、低級アルコキシ基またはハロアルキル基を示し、mは1乃至3の整数を示し、nは0乃至1の整数を示し、Aは酸素原子または硫黄原子を示す。）、または（III）（式（III）中、qは1乃至4の整数を示し、Bはヒドロキシ基、低級アルキルカルボニル基、置換基を有してもよいカルバモイル基、置換基を有してもよいアリール基、あるいは置換基を有してもよいアリールカルボニル基を示す。）である場合の本発明のピリドピリミジン誘導体の製造法は以下の通りである。

【0017】すなわち、下記に示す一般式（IV）で表される化合物を還元すると、一般式（V）で表される化合物を得ることができる。

【0018】

【化7】

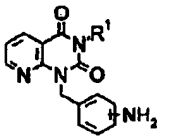


(IV)

【0019】式（IV）中、R^1は一般式（I）に示す意味と同じである。

【0020】

【化8】



(V)

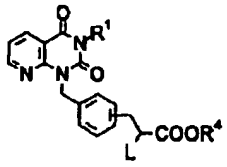
【0021】式（V）中、R^1は一般式（I）に示す意味と同じである。

【0022】還元の方法としては、接触還元、亜鉛－酢酸、鉄－酢酸、塩化スズ（II）二水和物による還元等が挙げられるが、好ましくは塩化スズ（II）二水和物が用いられる。溶媒としては、メタノール、エタノール等のアルコール類、テトラヒドロフラン等のエーテル類、酢酸等の有機酸類及びこれらの混合物が挙げられる。反応温度は通常、室温から加温下の範囲で行われる。例えば塩化スズ（II）二水和物を用いた還元における溶媒は通常、エタノールで反応温度は室温から還流温度の範囲で行われる。

【００２３】一般式（V）で表される化合物は、カラムクロマトグラフィーで精製の後、メイルバインアリレイション反応を行い一般式（VI）で表される化合物を生成する。反応は例えばＵＳＰ４４６１９０２号及びジャーナル オブ メディシナルケミストリー(Journal of Medicinal Chemistry)、32巻、421-428、1989年等に記載の方法に準じて行われる。すなわちメイルバインアリレイション反応は通常アセトン、水またはメタノール、エタノール等のアルコール類、好ましくはアセトンまたはアセトンと水の混合物を溶媒とし、常法によるジアゾ化後、酸の存在下、アクリル酸エステル類、次いで触媒量の第一銅塩を加えることにより行われる。

【００２４】

【化９】



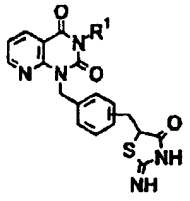
(VI)

【００２５】式（VI）中、R^1は一般式（I）に示す意味と同じであり、R^4はメチル基あるいはエチル基を示し、Lは塩素、臭素等のハロゲン原子を示す。

【００２６】次に一般式（VI）で表わされる化合物を酢酸ナトリウムの存在下、チオ尿素と反応させると一般式（VII）で表される化合物を得ることができる。

【００２７】

【化１０】



(VII)

【００２８】式（VII）中、R^1は一般式（I）に示す意味と同じである。

【００２９】最後に、一般式（VII）で表わされる化合物を酸加水分解することにより、目的とする一般式（I）で表される化合物を得る。

【００３０】酸加水分解反応に好適な酸としては、ギ酸、酢酸等の有機酸、硫酸、塩酸等の鉱酸が挙げられ、最も好ましくは塩酸を用いる。反応溶媒はエタノール、メタノール等のアルコール類、水、スルホラン、及びこれらの混合物が挙げられるが、好ましくは水とアルコール類の混合溶媒を用いる。

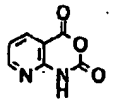
【００３１】前述した製造法で得られる本発明のピリドピリミジン誘導体は、例えば抽出、沈殿、分画クロマトグラフィー、分別結晶化、再結晶等の常法により単離、精製することができる。また、製造法において出発物質として用いられる一般式（IV）で表わされる化合物は、後述の方法１、方法２、あるいは方法３にて合成することができる。

【００３２】（方法１）一般式（IV）で表わされる化合物において、R^1は低級アルキル基、あるいは一般式（II）（式（II）中、R^2、R^3は水素原子、ハロゲン原子、低級アルキル基、低級アルコキシ基またはハロアルキル基を示し、mは１乃至３の整数を示し、nは０乃至１の整数を示し、Aは酸素原子または硫黄原子を示す。）または（III）（式（III）中、qは１乃至４の整数を示し、Bは低級アルキルカルボニル基、置換基を有してもよいアリール基、あるいは置換基を有してもよいアリールカルボニル基を示す。）である場合の化合物の合成法は以下の通りである。

【００３３】すなわち、下記に示す化合物（VIII）と一般式（IX）で表わされる化合物とを塩基性条件下反応させると化合物（X）が得られる。

【００３４】

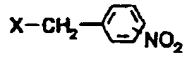
【化１１】



(VIII)

【００３５】

【化１２】

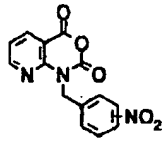


(IX)

【００３６】式（IX）中、Xは塩素、臭素、ヨウ素等のハロゲン原子を示す。

【００３７】

【化１３】



(X)

【００３８】この際の塩基としては、水素化ナトリウム等のアルカリ金属水素化物、水素化カルシウム等のアルカリ土類金属水素化物、炭酸ナトリウム、炭酸カリウム等のアルカリ金属炭酸塩、炭酸水素ナトリウム、炭酸水素カリウム等のアルカリ金属炭酸水素塩、トリエチルアミン、ジイソプロピルエチルアミン等のトリアルキルアミン、ピリジン、ルチジン、ピコリン、4-ジメチルアミノピリジン等のピリジン化合物等が挙げられ、好ましくは、水素化ナトリウムが用いられる。

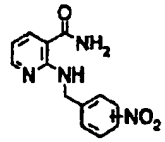
【００３９】上記の反応は通常種々の溶媒、例えばジクロロメタン、ピリジン、Ｎ,Ｎ－ジメチルホルムアミド、テトラヒドロフラン、アセトン、ジメチルスルホキシド等のような反応に悪影響を及ぼさない慣用の溶媒またはそれらの混合物中で行われる。特に好ましい溶媒と

しては、N,N-ジメチルホルムアミドが挙げられる。反応温度は通常は冷却下から加温下の範囲で反応が行われる。例えば塩基として水素化ナトリウムを用いた場合、反応温度は0℃から室温が好ましい。

【0040】次に、化合物（X）を濃アンモニア水で開環させることにより、化合物（XI）を得ることができる。

【0041】

【化14】

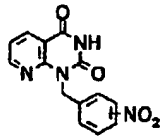


(XI)

【0042】次に、公知の方法（特開昭62－96478号、およびジャーナル オブ メディシナルケミストリー(Journal of Medicinal Chemistry)、34巻、1492-1503、1991年参照]に準じて、化合物（XI）にカルボニル化剤を作用させることにより、化合物（XII）を得ることができる。

【0043】

【化15】



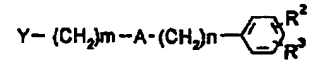
(XII)

【0044】上記の反応においてカルボニル化剤としては、1,1'-カルボニルジイミダゾール、ホスゲン、ビス（トリクロロメチル）カーボネート等が挙げられ、好ましくは1,1'-カルボニルジイミダゾールが用いられる。反応は好ましくは無溶媒で行われるが、反応に悪影響を及ぼさない慣用の溶媒、例えばクロロホルム、ジメチルホルムアミド等を用いることもできる。反応温度は通常加温下で行われる。

【0045】更に、化合物（XII）と一般式（XIII）あるいは（XIV）で表される化合物とを塩基性条件下反応させると、一般式（IV）で表される化合物を得る。

【0046】

【化16】

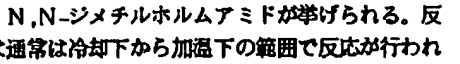


(XIII)

【0047】式（XIII）中、Yは塩素、臭素、ヨウ素等のハロゲン原子を示し、R^2、R^3は水素原子、ハロゲン原子、低級アルキル基、低級アルコキシ基またはハロアルキル基を示し、mは1乃至3の整数を示し、nは0乃至1の整数を示し、Aは酸素原子または硫黄原子を示す。

【0048】

【化17】



(XIV)

【0049】式（IV）中、Zは塩素、臭素、ヨウ素等のハロゲン原子を示し、qは1乃至4の整数を示し、Bは低級アルキルカルボニル基、置換基を有してもよいアリール基、あるいは置換基を有してもよいアリールカルボニル基を示す。

【0050】この際の塩基としては、水素化ナトリウム等のアルカリ金属水素化物、水素化カルシウム等のアルカリ土類金属水素化物、炭酸ナトリウム、炭酸カリウム等のアルカリ金属炭酸塩、炭酸水素ナトリウム、炭酸水素カリウム等のアルカリ金属炭酸水素塩、トリエチルアミン、ジイソプロピルエチルアミン等のトリアルキルアミン、ピリジン、ルチジン、ピコリン、4－ジメチルアミノピリジン等のピリジン化合物等が挙げられ、好ましくは、炭酸カリウムが用いられる。

【0051】（方法2）一般式（IV）で表わされる化合物において、R^1が上記方法1と同様である場合の化合物の別の合成法は以下の通りである。すなわち、化合物（VIII）と一般式（XV）で表わされる化合物とを塩基性条件下反応させると一般式（XVI）で表わされる化合物が得られる。

【0052】

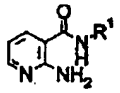
【化18】

(XV)

【0053】式（XV）中、R^1は一般式（I）に示す意味と同じである。]

【0054】

【化19】



(XVI)

【0055】式（XVI）中、R^1は一般式（I）に示す意味と同じである。]

【0056】この際の塩基としては、トリエチルアミン、N,N－ジイソプロピルエチルアミン、等のトリアルキルアミン類、ピリジン、ルチジン、ピコリン、4－ジメチルアミノピリジン等のピリジン化合物類が挙げられ、好ましくは、トリエチルアミンが用いられる。

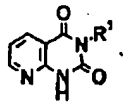
【0057】反応溶媒は、ジクロロメタン、トルエン、ピリジン、N,N－ジメチルホルムアミド、テトラヒドロフラン、アセトン等のような反応に悪影響を及ぼさない慣用の溶媒またはそれらの混合物中で行なわれ、好ましくはトルエンもしくはN,N－ジメチルホルムアミドが挙げられる。反応温度は通常は冷却下から還流下の範囲で行なわれる。

【0058】次に、前述の方法1に準じて、一般式（XV

I）で表わされる化合物にカルボニル化剤を作用させることにより、一般式（XVII）で表わされる化合物が得られる。

【0059】

【化20】



(XVII)

【0060】式（XVII）中、R^1は一般式（I）に示す意味と同じである。

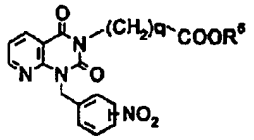
【0061】更に、前述の方法1に準じて、一般式（XVII）で表わされる化合物と一般式（）で表わされる化合物とを、アルカリ条件下反応させると、一般式（IV）で表わされる化合物を得る。

【0062】（方法3）一般式（IV）で表わされる化合物において、R^1は一般式（III）（式（III）中、qは1乃至4の整数を示し、Bは置換基を有してもよいカルバモイル基を示す。）である場合の化合物の合成法は以下の通りである。

【0063】すなわち、前述の方法1あるいは方法2に準じて合成した一般式（XVIII）で表わされる化合物を塩基性条件下エステルの加水分解を行なうと、一般式（XIX）で表わされる化合物を得る。

【0064】

【化21】

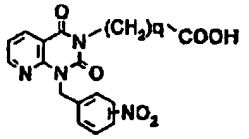


(XVIII)

【0065】式（XVIII）中、qは一般式（III）に示す意味と同じであり、R^5はメチル基あるいはエチル基を示す。

【0066】

【化22】



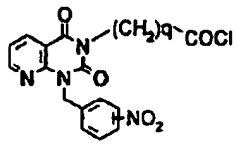
(XIX)

【0067】式（XIX）中、qは一般式（III）に示す意味と同じである。

【0068】次に、一般式（XIX）で表わされる化合物にハロゲン化試薬を作用させることにより、一般式（XX）で表わされる酸ハロゲン化物を得ることができる。

【0069】

【化23】



(XX)

【0070】式（XX）中、qは一般式（III）に示す意味と同じである。

【0071】この際のハロゲン化試薬としては、塩化ホスホリル、塩化チオニル、五塩化リン、三塩化リン等が挙げられるが、好ましくは塩化チオニルを用いる。

【0072】最後に、一般式（XX）表わされる化合物に、塩基性条件下、一般式（XXI）で表わされるアミノ化合物、あるいは一般式（XXII）表わされるピペラジニル化合物を反応させ、一般式（IV）で表わされる化合物を得た。

【0073】

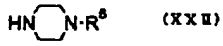
【化24】

(XXI)

【0074】式（XXI）中、R^6、R^7はそれぞれ独立して低級アルキル基を示す。

【0075】

【化25】



(XXII)

【0076】式（XXII）中、R^8は低級アルキル基を示す。

【0077】本発明のピリドピリミジン誘導体は優れた血糖降下作用とAR阻害作用を有しており、糖尿病合併症の予防ならびに治療において有効である。本発明の化合物を糖尿病合併症の予防ならびに治療を目的としてヒトに投与する場合はこれを内用、外用または局所投与に適した有機または無機の補助成分と共に固形製剤、半固形製剤または液状製剤として、経口的あるいは非経口的に投与可能である。その投与形態としては、経口製剤として錠剤、散剤、顆粒剤、カプセル剤、乳剤、懸濁剤、シロップ剤、非経口製剤としては座剤、注射剤、点滴剤、外用剤などとすることができる。

【0078】投与量は症状の程度、患者の年齢、疾病の進度などによって著しく異なるが、通常一日あたり0.01mg/kg乃至200mg/kg、好ましくは0.05mg/kg乃至50mg/kg、より好ましくは0.1mg/kg乃至10mg/kgの割合で、一日一乃至数回に分けて投与することができる。

【0079】製剤化にあたっては通常の製剤担体を用い、当該技術分野における常法によってこれをなすことができる。すなわち、経口的固形製剤を製造する場合は、主薬に賦形剤および必要に応じて結合剤、崩壊剤、

滑沢剤、着色剤、矯味剤、矯臭剤、などを加えた後、常法に従い錠剤、散剤、顆粒剤、カプセル剤、被覆製剤などとする。賦形剤としては、例えば乳糖、コーンスターチ、白糖、ブドウ糖、ソルビット、結晶セルロース、二酸化ケイ素などが用いられる。結合剤としては、例えばポリビニルアルコール、ポリビニルエーテル、エチルセルロース、メチルセルロース、アラビアゴム、トラガント、ゼラチン、シェラック、ヒドロキシプロピルスターチ、ポリビニルピロリドンなどが用いられる。崩壊剤としては、例えば澱粉、寒天、ゼラチン末、結晶セルロース、炭酸カルシウム、炭酸水素ナトリウム、クエン酸カルシウム、デキストリン、ペクチンなどが用いられる。滑沢剤としては、例えばステアリン酸マグネシウム、タルク、ポリエチレングリコール、シリカ、硬化植物油などが用いられる。着色剤としては、医薬品への添加が許可されているものが用いられる。矯味剤、矯臭剤としては、例えばココア末、ハッカ油、ハッカ脳、竜脳、芳香酸、ケイ皮末などが用いられる。これらの錠剤、顆粒剤に糖衣、ゼラチン衣、その他必要に応じて適宜コーティングを施すことは何ら差し支えない。注射剤を調製する場合には必要に応じて主薬にpH調製剤、緩衝剤、安定化剤、可溶化剤などを添加し常法により、皮下、筋肉内、静脈内用注射剤とする。

【００８０】

【実施例】以下、実施例をもって本発明を更に詳細に説明するが、本発明は何らこれらに限定されるものではない。なお、実施例中に述べる化合物の物性値は、プロトン核磁気共鳴スペクトル（ＮＭＲ）は、Varian Unity 400（400MHz）を用いて測定した。

【００８１】（実施例１）

１－｛４－〔（２,４－ジオキソチアゾリジン－５－イル）メチル〕ベンジル｝－３－〔２－（ベンジルオキシ）エチル〕－２,４－ジオキソ－１,２,３,４－テトラヒドロピリド［２,３－ｄ］ピリミジン（ＳＳＫ）の合成法

（Ａ）　１,２－ジヒドロ－２,４－ジオキソピリド［２,３－ｄ］－３,１－オキサジンおよび１,２－ジヒドロ－２,４－ジオキソピリド［３,２－ｄ］－３,１－オキサジンの合成法

２,３－ピリジンジカルボン酸無水物（４０.０ｇ，０.２７mol）のクロロホルム懸濁液（１８０ml，エタノールフリー）にトリメチルシリルアジド（３７.４ml，０.２８mol）を加え、反応が開始するまで注意深く加熱した。最初の急激な窒素の発生がおさまった後、更に加熱還流を行った。氷冷下にて反応液にエタノール（１５.８ml，０.２７mol）を加え、更に１５分間撹拌した後析出した沈殿を濾取、乾燥した。この沈殿物をアセトニトリル（３６０ml）中にて１時間加熱還流した後析出した沈殿を濾取し、乳白色粉末状の標記化合物（３４.０ｇ，７７%）を混合物として得た。尚、１,２－ジヒドロ－２,４－ジオキソピリド［２,３－ｄ］－３,１－オキサジンおよび１,２－ジヒドロ－２,４－ジオキソピリド［３,２－ｄ］－３,１－オキサジンの生成比は、４００MHzNMRより４：１であった。

【００８２】（Ｂ）　１,２－ジヒドロ－１－（４－ニトロベンジル）－２,４－ジオキソピリド［２,３－ｄ］－３,１－オキサジンの合成法

ｎ－ヘキサンで洗浄した水素化ナトリウム（６.４ｇ，０.１６mol）のＮ,Ｎ－ジメチルホルムアミド懸濁液（２００ml）中に１,２－ジヒドロ－２,４－ジオキソピリド［２,３－ｄ］－３,１－オキサジンおよび１,２－ジヒドロ－２,４－ジオキソピリド［３,２－ｄ］－３,１－オキサジンの混合物（２４.０ｇ，０.１５mol）を氷冷下少量ずつ加え、室温にて３０分間撹拌した後、ニトロベンジルブロミド（３４.８ｇ，０.１６mol）のジメチルホルムアミド溶液（８０ml）を氷冷下滴下し、更に室温にて３時間撹拌した。反応液を氷水に注ぎ析出した沈殿を濾取、乾燥し、薄桃色粉末状の標記粗化合物（３７.６ｇ，８６%）を得た。

【００８３】（Ｃ）　２－〔Ｎ－（４－ニトロベンジル）〕アミノニコチン酸アミドの合成法

１－（４－ニトロベンジル）－２,４－ジオキソピリド［２,３－ｄ］－３,１－オキサジン（３７.６ｇ，０.１３mol）のＮ,Ｎ－ジメチルホルムアミド懸濁液（２４０ml）中に、氷冷下、２５%アンモニア水（６０ml）を滴下し１時間撹拌した。反応液を２Ｎ塩酸で中和して析出した沈殿を濾過、水洗、乾燥し、黄土色粉末状の標記粗化合物（２７.２ｇ，８０%）を得た。

【００８４】（Ｄ）　１－（４－ニトロベンジル）－２,４－ジオキソ－１,２,３,４－テトラヒドロピリド［２,３－ｄ］ピリミジンの合成法

２－〔Ｎ－（４－ニトロベンジル）〕アミノニコチン酸アミド（２７.０ｇ，９９mmol）および１,１'－カルボニルジイミダゾール（３２.２ｇ，１９８mmol）を１３０℃にて融解させ、更に３０分間撹拌した。反応液を冷却し生じた固体をエタノールを加えて洗浄、濾取、乾燥した後、茶色粉末状の標記化合物（２４.０ｇ，８１%）を得た。

【００８５】（Ｅ）　１－（４－ニトロベンジル）－３－〔２－（ベンジルオキシ）エチル〕－２,４－ジオキソ－１,２,３,４－テトラヒドロピリド［２,３－ｄ］ピリミジンの合成法

１－（４－ニトロベンジル）－２,４－ジオキソ－１,２,３,４－テトラヒドロピリド［２,３－ｄ］ピリミジン（２.７９ｇ，９.４mmol）、２－（ベンジルオキシ）エチルブロミド（２.２１ｇ，１０.３mmol）および炭酸カリウム（１.９４ｇ，１４.０mmol）にＮ,Ｎ－ジメチルホルムアミド（１０ml）を加えて、６０℃で２時間撹拌した。反応液を水で希釈して酢酸エチルで抽出し、有機層を飽和食塩水で洗浄した後、無水硫酸ナトリウムで

乾燥して溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、淡黄色アモルファス状の標記化合物（3.68g，91%）を得た。

【0086】（F） 1－（4－アミノベンジル）－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－（4－ニトロベンジル）－3－〔2－（ベンジルオキシ）エチル〕－2,4-ジオキソ-1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（8.00g，18.5mmol）をエタノール（40ml）に溶解させ、塩化スズ（II）二水和物（16.7g，74.0mmol）を加えて、1時間還流した。反応液を水で希釈して2N水酸化ナトリウム水溶液でアルカリ性とした後、酢酸エチルで抽出した。有機層を飽和食塩水で洗浄して無水硫酸ナトリウムで乾燥した後、溶媒を減圧留去して、淡黄色アモルファス状の標記粗化合物（7.35g，99%）を得た。

【0087】（G） 1－〔4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル〕－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－（4－アミノベンジル）－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（3.48g，8.65mmol）のアセトン溶液（12ml）に蒸留水（3ml）、47%臭化水素酸（3ml，25.9mmol）を加えた後、5℃以下にて亜硝酸ナトリウム（0.66g，9.51mmol）の水溶液（3ml）を滴下し、30分間撹拌した。更にアクリル酸エチル（5.63ml，51.9mmol）を加え、反応液を40℃とした後、酸化銅（I）（0.13g，0.87mmol）を少量ずつ加えた。反応液を水で希釈して酢酸エチルで抽出した。有機層を飽和食塩水で洗浄し、無水硫酸ナトリウムで乾燥した後、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、黄色油状の標記化合物（3.54g，72%）を得た。

【0088】（H） 1－〔4－〔（2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－〔4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル〕－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（8.46g，14.9mmol）、チオ尿素（1.25g，16.4mmol）および酢酸ナトリウム（1.35g，16.4mmol）のエタノール溶液（70ml）を一夜還流した。反応液を冷却して析出した沈殿を濾取した後、エタノールで洗浄して、白色粉末状の標記粗化合物（7.30g，定量的）を得た。

【0089】（I） 1－〔4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－〔4－〔（2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（4.92g，10.2mmol）のエタノール溶液（100ml）に2N塩酸（50ml）を加えて一夜還流した。エタノールを留去した後、水で希釈して酢酸エチルで抽出した。有機層を飽和食塩水で洗浄して無水硫酸ナトリウムで乾燥した後、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、白色アモルファス状の標記化合物（4.08g，83%）を得た。この化合物のNMRデータを以下に示す。

【0090】^{1}H－NMR（CDCl$_3$，400MHz，TMS） δ：3.07（1H,dd,J＝10.0，14.0Hz），3.48（1H,dd,J＝4.0，14.0Hz），3.79（2H,d，J＝6.0Hz），4.37（2H,d,J＝6.0Hz），4.46（1H,dd,J＝4.0，10.0Hz），4.54（2H,s），5.52（2H,s），7.12（2H,d,J＝8.0Hz），7.18～7.26（6H,m），7.45（2H,d,J＝8.0Hz），8.45（1H,dd,J＝1.6，7.7Hz），8.66（1H,dd,J＝1.6，4.8Hz）

【0091】（実施例2）

1－〔4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－（2－ヒドロキシエチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（SSL）の合成法

（A） 1－〔4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－（2－ヒドロキシエチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－〔4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（1.00g，2.0mmol）を塩化メチレン（5ml）に溶解させ、窒素雰囲気下、0℃で三臭化ホウ素（2ml）を滴下し、室温で1時間撹拌した。反応液にメタノールを加えた後、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、白色アモルファス状の標記化合物（0.68g，86%）を得た。この化合物のNMRデータを以下に示す。

【0092】^{1}H－NMR（CDCl$_3$，DMSO－d$_6$，400MHz，TMS） δ：3.06（1H，d

d，J＝10.1，14.3Hz），3.44（1H,dd，J＝3.8，14.3Hz），3.87（2H,d,J＝5.5Hz），4.31（2H,t,J＝5.5Hz），4.43（1H,dd,J＝3.8，10.1Hz），5.54（2H，s），7.16（2H,d,J＝8.1Hz），7.24（1H,dd,J＝4.8，7.9Hz），7.44（2H,d,J＝8.1Hz），8.46（1H,dd,J＝2.0，7.9Hz），8.68（1H,dd,J＝2.0，4.8Hz）

【0093】（実施例3）

1－｛4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル｝－3－（4－フェニルブチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（SSM）の合成法

（A） 1－（4－ニトロベンジル）－3－（4－フェニルブチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（E）と同様に、実施例1（D）で得たピリドピリミジン2.00g（6.70mmol）、4－フェニルブチルブロミド1.57g（7.38mmol）、炭酸カリウム1.39g（10.05mmol）、N,N－ジメチルホルムアミド7mlを用いて、白色アモルファス状の標記化合物（1.54g，53%）を得た。

【0094】（B） 1－（4－アミノベンジル）－3－（4－フェニルブチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（F）と同様に、実施例3（A）で得たニトロ体1.54g（3.58mmol）、塩化スズ（II）二水和物3.23g（14.31mmol）、エタノール20mlを用いて、薄黄色アモルファス状の標記化合物（1.23g，86%）を得た。

【0095】（C） 1－〔4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル〕－3－（4－フェニルブチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（G）と同様に、実施例3（B）で得たアミン体1.23g（3.07mmol）、47%臭化水素酸3.23g（14.31mmol）、亜硝酸ナトリウム233mg（3.38mmol）、アセトン5ml、アクリル酸エチル2.00ml（18.42mmol）、酸化銅（I）46mg（0.31mmol）を用いて、白色アモルファス状の標記化合物（0.53g，31%）を得た。

【0096】（D） 1－｛4－〔（2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル｝－3－（4－フェニル）ブチル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（H）と同様に、実施例3（C）で得たハロエステル体320mg（0.57mmol）、チオ尿素47mg（0.62mmol）、酢酸ナトリウム51mg（0.62mmol）、エタノール5mlを用いて、白色アモルファス状の標記化合物（0.16g，58%）を得た。

【0097】（E） 1－｛4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル｝－3－（4－フェニルブチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（1）と同様に、実施例3（D）で得たイミン体0.10g（0.57mmol）、2N塩酸1.0ml（0.62mmol）、エタノール2mlを用いて、白色アモルファス状の標記化合物（0.08g，80%）を得た。この物のNMRデータを以下に示す。

【0098】^{1}H－NMR（CDCl$_3$，400Mhz，TMS） δ：1.67～1.77（2H,m），2.65（2H,t,J＝7.0Hz），3.07（1H,dd,J＝9.9，14.0Hz），3.49（1H,dd,J＝3.8，14.0Hz），4.11（2H,t,J＝7.0Hz），4.47（1H,dd,J＝3.8，9.9Hz），5.53（2H，s），7.14（2H,d,J＝8.1Hz），7.16～7.27（6H,m），7.44（2H,d,J＝8.1Hz），8.46（1H,dd,J＝1.8，7.9Hz），8.65（1H,dd,J＝1.8，4.8Hz）

【0099】（実施例4）

1－｛4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル｝－3－（4－ピフェニルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（SSN）の合成法

（A） 1－（4－ニトロベンジル）－3－（4－ピフェニルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（E）と同様に、実施例1（D）で得たピリドピリミジン100mg（0.33mmol）、4－（ブロモメチル）ピフェニル92mg（0.37mmol）、炭酸カリウム70mg（0.50mmol）、N,N－ジメチルホルムアミド1mlを用いて、白色アモルファス状の標記化合物（65mg，42%）を得た。

【0100】（B） 1－（4－アミノベンジル）－3－（4－ピフェニルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（F）と同様に、実施例4（A）で得たニトロ体0.75g（1.61mmol）、塩化スズ（II）二水和物1.45g（6.44mmol）、エタノール10mlを用いて、薄黄色アモルファス状の標記化合物（0.62g，89%）を得た。

【0101】（C） 1－〔4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル〕－3－（4－ピフェニルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（G）と同様に、実施例4（B）で得たアミン

体622mg（1.43mmol）、47%臭化水素酸0.5ml（4.29mmol）、亜硝酸ナトリウム108mg（3.38mmol）、アセトン5ml、アクリル酸エチル0.93ml（8.58mmol）、酸化銅（I）20mg（0.14mmol）を用いて、白色アモルファス状の標記化合物（0.48g，56%）を得た。

【0102】（D）　1－{4－〔（2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル}－3－（4－ピフェニルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（H）と同様に、実施例4（C）で得たハロエステル体470mg（0.79mmol）、チオ尿素66mg（0.86mmol）、酢酸ナトリウム71mg（0.86mmol）、エタノール5mlを用いて、白色アモルファス状の標記粗化合物（0.40g，定量的）を得た。

【0103】（E）　1－{4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル}－3－（4－ピフェニルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（I）と同様に、実施例4（D）で得たイミン体0.40g（0.79mmol）、2N塩酸4.0ml、エタノール8mlを用いて、白色アモルファス状の標記化合物（400mg，98%）を得た。この物のNMRデータを以下に示す。

【0104】^{1}H－NMR（CDCl$_3$，400MHz，TMS）　δ：3.09（1H,dd,J＝9.9，14.1Hz），3.49（1H,dd,J＝3.8，14.1Hz），4.48（1H,dd,J＝3.8，9.9Hz），5.31（2H,s），5.56（2H,s），7.22（1H,dd,J＝4.8，7.7Hz），7.15～7.60（13H,m），8.49（1H,dd,J＝2.0，7.7Hz），8.66（1H,dd,J＝2.0，4.8Hz）

【0105】（実施例5）

1－{4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル}－3－〔2－（フェニルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（SSO）の合成法

（A）　1－（4－ニトロベンジル）－3－〔2－（フェニルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（E）と同様に、実施例1（D）で得たピリドピリミジン2.00g（6.70mmol）、β－ブロモフェネトール1.48g（7.37mmol）、炭酸カリウム1.39g（10.05mmol）、N,N－ジメチルホルムアミド14mlを用いて、白色アモルファス状の標記化合物（1.70g，60%）を得た。

【0106】（B）　1－（4－アミノベンジル）－3－〔2－（フェニルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（F）と同様に、実施例5（A）で得たニトロ体1.70g（4.06mmol）、塩化スズ（II）二水和物3.67g（16.25mmol）、エタノール20mlを用いて、薄黄色アモルファス状の標記化合物（1.53g，97%）を得た。

【0107】（C）　1－〔4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル〕－3－〔2－（フェニルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（G）と同様に、実施例5（B）で得たアミン体1.53g（3.94mmol）、47%臭化水素酸1.4ml（11.82mmol）、亜硝酸ナトリウム300mg（4.33mmol）、アセトン8ml、アクリル酸エチル2.56ml（23.64mmol）、酸化銅（I）58mg（0.39mmol）を用いて、白色アモルファス状の標記化合物（1.44g，66%）を得た。

【0108】（D）　1－{4－〔（2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル}－3－〔2－（フェニルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（H）と同様に、実施例5（C）で得たハロエステル体1.44g（2.61mmol）、チオ尿素218mg（2.87mmol）、酢酸ナトリウム235mg（2.87mmol）、エタノール10mlを用いて、白色アモルファス状の標記粗化合物（1.18g，96%）を得た。

【0109】（E）　1－{4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル}－3－〔2－（フェニルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（I）と同様に、実施例5（D）で得たイミン体1.18g（2.51mmol）、2N塩酸12.5ml、エタノール25mlを用いて、白色アモルファス状の標記化合物（1.14g，97%）を得た。この物のNMRデータを以下に示す。

【0110】^{1}H－NMR（CDCl$_3$，400MHz，TMS）　δ：3.09（1H,dd,J＝9.9，14.1Hz），3.49（1H,dd,J＝3.7，14.1Hz），4.28（2H,t,J＝6.0Hz），4.48（1H,dd,J＝3.7，9.9Hz），4.54（2H,d,J＝6.0Hz），5.55（2H,s），6.85～6.93（3H,m），7.15（2H,d,J＝8.1Hz），7.21～7.23（3H,m），7.46（2H,d,J＝8.1Hz），8.48（1H,dd,J＝2.0，7.7Hz），8.

67（1H,dd,J＝2.0，4.8Hz）

【0111】（実施例6）

1－{3－〔(2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル}－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（SSP）の合成法

（A） 1－（3－ニトロベンジル）－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（E）と同様に、実施例1（D）で得たピリドピリミジン1.70g（5.70mmol）、2－（ベンジルオキシ）エチルブロミド1.35g（6.27mmol）、炭酸カリウム1.18g（8.55mmol）、N,N－ジメチルホルムアミド12mlを用いて、白色アモルファス状の標記化合物（2.53g，定量的）を得た。

【0112】（B） 1－（3－アミノベンジル）－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（F）と同様に、実施例5（A）で得たニトロ体2.53g（5.70mmol）、塩化スズ（II）二水和物5.14g（22.80mmol）、エタノール12mlを用いて、薄黄色非晶状の標記化合物（2.31g，定量的）を得た。

【0113】（C） 1－〔3－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル〕－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（G）と同様に、実施例6（B）で得たアミン体2.31g（5.74mmol）、47%臭化水素酸1.9ml（17.22mmol）、亜硝酸ナトリウム435mg（6.31mmol）、アセトン20ml、アクリル酸エチル3.73ml（34.44mmol）、酸化銅（I）85mg（0.57mmol）を用いて、無色油状の標記化合物（1.42g，44%）を得た。

【0114】（D） 1－{3－〔(2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル}－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（H）と同様に、実施例6（C）で得たハロエステル体1.40g（2.47mmol）、チオ尿素207mg（2.72mmol）、酢酸ナトリウム223mg（2.72mmol）、エタノール12mlを用いて、白色アモルファス状の標記粗化合物（1.10g，87%）を得た。

【0115】（E） 1－{3－〔(2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル}－3－〔2－（ベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（I）と同様に、実施例6（D）で得たイミン体1.10g（2.13mmol）、2N塩酸13ml、エタノール25mlを用いて、白色アモルファス状の標記化合物（1.01g，92%）を得た。この物のNMRデータを以下に示す。

【0116】^{1}H－NMR（CDCl$_3$，400MHz，TMS） δ：3.17（1H,dd,J＝8.4，14.1Hz），3.35（1H,dd,J＝4.0，14.1Hz），3.79～3.87（2H,m），4.36～4.39（2H,m），4.45（1H,dd,J＝4.0，8.4Hz），4.57（2H,s），5.53（2H,q,J＝14.3Hz），7.09（1H,d,J＝7.7Hz），7.20～7.31（8H,m），7.42（1H,d,J＝7.7Hz），8.47（1H,dd,J＝2.0，7.7Hz），8.67（1H,dd,J＝2.0，4.8Hz）

【0117】（実施例7）

1－{4－〔(2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル}－3－〔3－（フェニルオキシ）プロピル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（SSQ）の合成法

（A） 1－（4－ニトロベンジル）－3－〔3－（フェニルオキシ）プロピル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（E）と同様に、実施例1（D）で得たピリドピリミジン2.50g（8.38mmol）、3－（フェニルオキシ）プロピルブロミド1.98g（9.22mmol）、炭酸カリウム1.74g（12.57mmol）、N,N－ジメチルホルムアミド20mlを用いて、白色アモルファス状の標記化合物（2.53g，70%）を得た。

【0118】（B） 1－（4－アミノベンジル）－3－〔3－（フェニルオキシ）プロピル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（F）と同様に、実施例7（A）で得たニトロ体2.53g（5.85mmol）、塩化スズ（II）二水和物5.28g（23.40mmol）、エタノール20mlを用いて、薄黄色アモルファス状の標記化合物（2.48g，定量的）を得た。

【0119】（C） 1－〔4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル〕－3－〔3－（フェニルオキシ）プロピル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（G）と同様に、実施例7（B）で得たアミン体2.48g（6.16mmol）、47%臭化水素酸2.0

6ml（18.49mmol）、亜硝酸ナトリウム468mg（6.78mmol）、アセトン20ml、アクリル酸エチル4.00ml（36.98mmol）、酸化銅（I）92mg（0.62mmol）を用いて、白色アモルファス状の標記化合物（2.27g，65%）を得た。

【0120】（D） 1－{4－〔（2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル}－3－〔3－（フェニルオキシ）プロピル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（H）と同様に、実施例7（C）で得たハロエステル体2.25g（3.98mmol）、チオ尿素333mg（4.38mmol）、酢酸ナトリウム359mg（4.38mmol）、エタノール20mlを用いて、白色アモルファス状の標記粗化合物(1.88g，92%)を得た。

【0121】（E） 1－{4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル}－3－〔3－（フェニルオキシ）プロピル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（I）と同様に、実施例7（D）で得たイミン体1.88g（3.56mmol）、2N塩酸20ml、エタノール40mlを用いて、白色アモルファス状の標記化合物（1.72g，91%）を得た。この物のNMRデータを以下に示す。

【0122】^{1}H－NMR（CDCl$_3$，400MHz，TMS） δ：2.20～2.24（2H,m），3.08（1H,dd,J＝9.7，14.1Hz），3.48（1H,dd,J＝3.8，14.1Hz），4.07（2H,t,J＝7.0Hz），4.32（2H,t,J＝7.0Hz），4.47（1H,dd,J＝3.8，9.7Hz），5.52（2H,s），6.78～6.93（3H,m），7.13（2H,d，J＝8.1Hz），7.20～7.26（3H,m），7.44（2H,d,J＝8.1Hz），8.45（1H,dd,J＝2.0，7.5Hz），8.67（1H,dd,J＝2.0，4.8Hz）

【0123】（実施例8）

1－{4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル}－3－（2－ピリジンエチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（SSR）の合成法

（A） 1－（4－ニトロベンジル）－3－（2－ピリジンエチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（E）と同様に、実施例1（D）で得たピリドピリミジン2.50mg（8.38mmol）、2－ピリジンエチルブロミド1.72mg（9.22mmol）、炭酸カリウム1.74mg（12.57mmol）、N,N－ジメチルホルムアミド10mlを用いて、白色アモルファス状の標記化合物（3.18g，94%）を得た。

【0124】（B） 1－（4－アミノベンジル）－3－（2－ピリジンエチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（F）と同様に、実施例8（A）で得たニトロ体3.18g（7.88mmol）、塩化スズ（II）二水和物7.11g（31.53mmol）、エタノール50mlを用いて、薄黄色アモルファス状の標記粗化合物（1.00g，34%）を得た。

【0125】（C） 1－〔4－（2－ブロモ－2－エトキシカルボニル）ベンジル〕－3－（2－ピリジンエチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（G）と同様に、実施例8（B）で得たアミン体1.00g（2.68mmol）、47%臭化水素酸0.93ml（8.04mmol）、亜硝酸ナトリウム0.20g（2.95mmol）、アセトン10ml、アクリル酸エチル1.75ml（16.08mmol）、酸化銅（I）40mg（0.27mmol）を用いて、白色アモルファス状の標記化合物（0.57g，40%）を得た。

【0126】（D） 1－{4－〔（2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル}－3－（2－ピリジンエチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（H）と同様に、実施例8（C）で得たハロエステル体0.55g（1.02mmol）、チオ尿素86mg（1.13mmol）、酢酸ナトリウム93mg（1.13mmol）、エタノール5mlを用いて、白色アモルファス状の標記粗化合物（0.15g，30%）を得た。

【0127】（E） 1－{4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル}－3－（2－ピリジンエチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（I）と同様に、実施例8（D）で得たイミン体0.15g（0.31mmol）、2N塩酸3ml、エタノール6mlを用いて、白色アモルファス状の標記化合物（40mg，27%）を得た。この物のNMRデータを以下に示す。

【0128】^{1}H－NMR（DMSO－d$_6$，400MHz，TMS） δ：3.06～3.10（3H,m），3.89（1H,m），4.34（2H,t,J＝8.0Hz），4.83（1H,dd,J＝4.0，9.2Hz），5.42（2H,s），7.16～7.19（3H,m），7.23～7.27（3H,m），7.34（1H,dd,J＝4.8，7.9Hz），7.66（1H,dd,J＝1.8，7.6Hz），8.40～8.42（2H,m），8.69（1H,dd,J＝1.8，4.8Hz）

【0129】（実施例9）

1－{4－〔(2,4－ジオキソチアゾリジン－5－イル)メチル〕ベンジル}－3－{2－〔4－(イソプロピル)ベンジルオキシ〕エチル}－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジン(SSS)の合成法

(A) 1－(4－ニトロベンジル)－3－{2－〔4－(イソプロピル)ベンジルオキシ〕エチル}－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1(E)と同様に、実施例1(D)で得たピリドピリミジン2.50g(8.38mmol)、2－〔4－(イソプロピル)ベンジルオキシ〕エチルブロミド2.37g(9.22mmol)、炭酸カリウム1.74g(12.57mmol)、N,N－ジメチルホルムアミド20mlを用いて、白色アモルファス状の標記化合物(2.68g,67%)を得た。

〔0130〕(B) 1－(4－アミノベンジル)－3－{2－〔4－(イソプロピル)ベンジルオキシ〕エチル}－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1(F)と同様に、実施例9(A)で得たニトロ体2.68g(5.65mmol)、塩化スズ(II)二水和物5.10g(22.60mmol)、エタノール10mlを用いて、薄黄色アモルファス状の標記化合物(2.43g,97%)を得た。

〔0131〕(C) 1－〔4－(2－ブロモ－2－エトキシカルボニルエチル)ベンジル〕－3－{2－〔4－(イソプロピル)ベンジルオキシ〕エチル}－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1(G)と同様に、実施例9(B)で得たアミン体2.43g(5.47mmol)、47%臭化水素酸1.83ml(16.41mmol)、亜硝酸ナトリウム0.42g(6.01mmol)、アセトン20ml、アクリル酸エチル3.55ml(32.82mmol)、酸化銅(I)82mg(0.55mmol)を用いて、白色アモルファス状の標記化合物(1.90g,57%)を得た。

〔0132〕(D) 1－{4－〔(2－イミノ－4－オキソチアゾリジン－5－イル)メチル〕ベンジル}－3－{2－〔4－(イソプロピル)ベンジルオキシ〕エチル}－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1(H)と同様に、実施例9(C)で得たハロエステル体1.90g(3.12mmol)、チオ尿素261mg(3.43mmol)、酢酸ナトリウム281mg(3.43mmol)、エタノール15mlを用いて、白色アモルファス状の標記粗化合物(1.74g,定量的)を得た。

〔0133〕(E) 1－{4－〔(2,4－ジオキソチアゾリジン－5－イル)メチル〕ベンジル}－3－{2－〔4－(イソプロピル)ベンジルオキシ〕エチル}－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1(I)と同様に、実施例9(D)で得たイミン体1.74g(3.12mmol)、2N塩酸15ml、エタノール30mlを用いて、白色アモルファス状の標記化合物(1.10g,63%)を得た。この物のNMRデータを以下に示す。

〔0134〕^{1}H－NMR(CDCl$_3$,400MHz,TMS) δ:1.20(3H,s),1.22(3H,s),2.85(2H,m),3.08(1H,dd,J=9.9,14.1Hz),3.49(1H,dd,J=3.8,14.1Hz),3.78(2H,t,J=5.9Hz),4.37(2H,t,J=5.9Hz),4.37(2H,t,J=5.9Hz),4.47(1H,dd,J=3.8,9.9Hz),4.52(2H,s),5.53(2H,s),7.10～7.26(7H,m),7.45(2H,d,J=8.2Hz),8.46(1H,dd,J=2.0,7.9Hz),8.66(1H,dd,J=2.0,4.8Hz)

〔0135〕(実施例10)

1－{4－〔(2,4－ジオキソチアゾリジン－5－イル)メチル〕ベンジル}－3－(N,N-ジメチルカルバモイルメチル)－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジン(SSSJ)の合成法

(A) 1－(4－ニトロベンジル)－3－エトキシカルボニルメチル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

n－ヘキサンで洗浄した水素化ナトリウム(0.89g,22.13mmol)のN,N－ジメチルホルムアミド懸濁液(40ml)中に1－(4－ニトロベンジル)－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジン(6.00g,20.12mmol)を氷冷下少量ずつ加え、室温にて30分間撹拌した後、ブロモ酢酸エチル(2.45ml,22.13mmol)のN,N－ジメチルホルムアミド溶液(10ml)を氷冷下滴下し、更に室温にて3時間撹拌した。反応液を氷水に注ぎ析出した沈殿を濾取、乾燥した後、薄黄色粉末状の標記粗化合物(6.36g,82%)を得た。

〔0136〕(B) 1－(4－ニトロベンジル)－3－カルボキシメチル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

1－(4－ニトロベンジル)－3－エトキシカルボニルメチル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジン(6.36g,16.55mmol)をテトラヒドロフラン-メタノール(1:1)溶液(40ml)に溶解し、2当量の2N水酸化ナトリウム水溶液(16.5ml,33.10mmol)を加えて1時間撹拌した。反応液を1N塩酸を加えて酸性(pH3)として酢酸エチルで抽出した。有機層を飽和食塩水で洗浄して無水硫酸ナトリウムで乾燥した後、溶媒を減圧留去

した。残渣をシリカゲルカラムクロマトグラフィーで精製し、薄黄色アモルファス状の標記化合物（5.64g，96%）を得た。

【0137】（C） 1－（4－ニトロベンジル）－3－（N,N－ジメチルカルバモイルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－（4－ニトロベンジル）－3－カルボキシメチル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（2.50g，7.02mmol）に塩化チオニル（10ml）を加えて、外浴100℃で1時間撹拌した。放冷後、過剰の塩化チオニルを減圧留去して、黄色油状の酸クロリドを得た。続いて、ジメチルアミン塩酸塩（0.69g，8.42mmol）のクロロホルム溶液（15ml）に、トリエチルアミン（2.94ml，21.06mmol）を加えて、氷冷下、上記酸クロリドのクロロホルム溶液（5ml）を滴下し1時間撹拌した。反応液を水で希釈してクロロホルムで抽出した。有機層を飽和食塩水で洗浄して、無水硫酸ナトリウムで乾燥した後、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、薄黄色アモルファス状の標記化合物（1.66g，62%）を得た。

【0138】（D） 1－（4－アミノベンジル）－3－（N,N－ジメチルカルバモイルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－（4－ニトロベンジル）－3－（N,N－ジメチルカルバモイルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（1.66g，4.33mmol）をエタノール（15ml）に溶解させ、塩化スズ（II）二水和物（3.91g，17.32mmol）を加えて2時間還流した。反応液を水で希釈し、1N水酸化ナトリウム水溶液でアルカリ性としてクロロホルムで抽出した。有機層を飽和食塩水で洗浄して無水硫酸ナトリウムで乾燥した後、溶媒を減圧留去して、淡黄色アモルファス状の標記粗化合物（1.44g，94%）を得た。

【0139】（E） 1－［4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル］－3－（N,N－ジメチルカルバモイルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－（4－アミノベンジル）－3－（N,N－ジメチルカルバモイルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（1.44g，4.08mmol）のアセトン溶液（12ml）に蒸留水（3ml）、47%臭化水素酸（1.41ml，12.24mmol）を加えた後、5℃以下にて亜硝酸ナトリウム（0.31g，4.49mmol）の水溶液を滴下し、30分間撹拌した。更にアクリル酸エチル（2.65ml，24.48mmol）を加え、反応液を40℃とした後、酸化銅（I）（61mg，0.41mmol）を少量ずつ加えた。反応液を水で希釈して酢酸エチルで抽出した。有機層を飽和食塩水で洗浄して無水硫酸ナトリウムで乾燥した後、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、黄色油状の標記化合物（1.82g，86%）を得た。

【0140】（F） 1－｛4－［（2－イミノ－4－オキソチアゾリジン－5－イル）メチル］ベンジル｝－3－（N,N－ジメチルカルバモイルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－［4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル］－3－（N,N－ジメチルカルバモイルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（1.80g，3.48mmol）、チオ尿素（0.32g，4.17mmol）および酢酸ナトリウム（0.34g，4.17mmol）のエタノール溶液（20ml）を一晩還流した。反応液を冷却して析出した沈殿を濾取した後、エタノールで洗浄して、白色粉末状の標記粗化合物（1.10g，68%）を得た。

【0141】（G） 1－｛4－［（2,4－ジオキソチアゾリジン－5－イル）メチル］ベンジル｝－3－（N,N－ジメチルカルバモイルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

1－｛4－［（2－イミノ－4－オキソチアゾリジン－5－イル）メチル］ベンジル｝－3－（N,N－ジメチルカルバモイルメチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（1.10g，2.38mmol）のエタノール溶液（20ml）に2N塩酸（11ml）を加えて5時間還流した。反応液はエタノールを留去し、水で希釈し、酢酸エチルで抽出した。有機層を飽和食塩水で洗浄し、無水硫酸ナトリウムで乾燥し、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、白色アモルファス状の標記化合物（0.90g，81%）を得た。この物のNMRデータを以下に示す。

【0142】^{1}H－NMR（CDCl$_3$，400MHz，TMS） δ：2.99（3H,s），3.08（1H,dd,J＝9.7，14.1Hz），3.12（3H,s），3.46（1H,dd,J＝3.7，14.1Hz），4.47（1H,dd,J＝3.7，9.7Hz），4.90（2H,s），5.55（2H,s），7.15（2H,d,J＝8.3Hz），7.22（1H,dd,J＝4.8，7.7Hz），7.43（2H,d,J＝8.3Hz），8.46（1H,dd,J＝2.0，7.7Hz），8.46（1H,dd,J＝2.0，7.7Hz），8.67（1H,dd,J＝2.0，4.8Hz）

【0143】（実施例11）

1－{4－〔(2,4－ジオキソチアゾリジン－5－イル)メチル〕ベンジル}－3－〔(4－メチルピペラジン－1－イル)カルボニルメチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジン(SSSK)の合成法

(A) 1－(4－ニトロベンジル)－3－〔(4－メチルピペラジン－1－イル)カルボニルメチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例10(C)と同様に、実施例10(B)で得たカルボン酸2.50g(7.02mmol)、塩化チオニル5ml、1－メチルピペラジン0.93ml(8.42mmol)、トリエチルアミン1.96ml(14.04mmol)、クロロホルム20mlを用いて、薄黄色アモルファス状の標記化合物(2.20g,71%)を得た。

【0144】(B) 1－(4－アミノベンジル)－3－〔(4－メチルピペラジン－1－イル)カルボニルメチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例10(D)と同様に、実施例11(C)で得たニトロ体1.98g(4.31mmol)、塩化スズ(II)二水和物3.89g(17.24mmol)、エタノール20mlを用いて、黄色アモルファス状の標記化合物(1.74g,99%)を得た。

【0145】(C) 1－{4－(2－ブロモ－2－エトキシカルボニルエチル)ベンジル}－3－〔(4－メチルピペラジン－1－イル)カルボニルメチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例10(E)と同様に、実施例11(B)で得たアミン体1.81g(4.43mmol)、47%臭化水素酸1.54ml(13.29mmol)、亜硝酸ナトリウム0.34g(4.87mmol)、アセトン10ml、アクリル酸エチル2.88ml(26.58mmol)、酸化銅(I)65mg(0.44mmol)を用いて、薄黄色アモルファス状の標記化合物(1.42g,56%)を得た。

【0146】(D) 1－{4－〔(2－イミノ－4－オキソチアゾリジン－5－イル)メチル〕ベンジル}－3－〔(4－メチルピペラジン－1－イル)カルボニルメチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例10(F)と同様に、実施例11(C)で得たハロエステル体1.40g(2.45mmol)、チオ尿素220mg(2.93mmol)、酢酸ナトリウム240mg(2.93mmol)、エタノール10mlを用いて、白色アモルファス状の標記粗化合物(0.46g,36%)を得た。

【0147】(E) 1－{4－〔(2,4－ジオキソチアゾリジン－5－イル)メチル〕ベンジル}－3－〔(4－メチルピペラジン－1－イル)カルボニルメチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例10(G)と同様に、実施例11(D)で得たイミン体0.85g(1.63mmol)、2N塩酸8ml、エタノール10mlを用いて、薄黄色アモルファス状の標記化合物(0.11g,13%)を得た。この物のNMRデータを以下に示す。

【0148】^{1}H－NMR(CDCl$_3$,400MHz,TMS) δ:2.32(3H,s),2.43～2.53(4H,br),3.13(1H,dd,J=9.2,14.1Hz),3.41(1H,dd,J=3.7,14.1Hz),3.51～3.64(4H,br),4.47(1H,dd,J=3.7,9.2Hz),4.91(2H,s),5.55(2H,s),7.16(2H,d,J=8.0Hz),7.23(1H,dd,J=4.8,7.7Hz),7.44(2H,d,J=8.0Hz),8.46(1H,dd,J=2.0,7.7Hz),8.68(1H,dd,J=2.0,4.8Hz)

【0149】(実施例12)

1－{4－〔(2,4－ジオキソチアゾリジン－5－イル)メチル〕ベンジル}－3－(4－オキソペンチル)－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジン(SSSL)の合成法

(A) 1－(4－ニトロベンジル)－3－(4－オキソペンチル)－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

1－(4－ニトロベンジル)－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジン(4.00g,13.41mmol)、5－クロロ－2－ペンタノン(1.84ml,16.09mmol)、ヨウ化カリウム(2.67g,16.09mmol)および炭酸カリウム(3.71g,26.82mmol)にN,N－ジメチルホルムアミド(160ml)を加えて、60℃で2日間撹拌した。反応液を水で希釈して酢酸エチルで抽出した。有機層を飽和食塩水で洗浄して無水硫酸ナトリウムで乾燥した後、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、淡黄色アモルファス状の標記化合物(2.90g,57%)を得た。

【0150】(B) 1－(4－アミノベンジル)－3－(4－オキソペンチル)－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1(F)と同様に、実施例12(A)で得たニトロ体2.69g(7.01mmol)、塩化スズ(II)二水和物6.33g(28.04mmol)、エタノール25mlを用いて、薄黄色アモルファス状の標記化合物(2.45g,99%)を得た。

【0151】(C) 1－{4－(2－ブロモ－2－エトキシカルボニルエチル)ベンジル}－3－(4－オキソペンチル)－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1(G)と同様に、実施例12(B)で得たアミ

ン体1.43g（4.06mmol）、47%臭化水素酸1.41ml（12.18mmol）、亜硝酸ナトリウム0.31g（4.47mmol）、アセトン10ml、アクリル酸エチル2.64ml（24.36mmol）、酸化銅（I）61mg（0.41mmol）を用いて、黄色油状の標記化合物（1.50g，72%）を得た。

【0152】（D） 1－{4－[（2,4－ジオキソチアゾリジン－5－イル）メチル]ベンジル}－3－（4－オキソペンチル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1（H）と同様に、実施例12（C）で得たハロエステル体1.47g（2.85mmol）、チオ尿素260mg（3.41mmol）、酢酸ナトリウム280mg（3.41mmol）、エタノール15mlを用いて、引き続き、実施例1（I）と同様に、2N塩酸10mlを用いて、白色アモルファス状の標記化合物（1.07g，80%）を得た。この物のNMRデータを以下に示す。

【0153】^{1}H－NMR（CDCl$_3$，400MHz，TMS） δ：1.98（2H,dt,J=7.1Hz），2.11（3H，s），2.52（2H,t,J=7.1Hz），3.11（1H,dd,J=9.5，14.1Hz），3.48（1H,dd，J=3.9，14.1Hz），4.10（2H,t,J=7.1Hz），4.49（1H,dd,J=3.9，9.5Hz），5.54（2H,s），7.16（2H,d,J=8.0Hz），7.22（1H,dd,J=4.8，7.7Hz），7.45（2H,d,J=8.0Hz），8.46（1H,dd,J=1.8，7.7Hz），8.67（1H,dd,J=1.8，4.8Hz）

【0154】（実施例13）

1－{4－[（2,4－ジオキソチアゾリジン－5－イル）メチル]ベンジル}－3－フェナシル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジン（SSSM）の合成法

（A） 1－（4－ニトロベンジル）－3－フェナシル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

n－ヘキサンで洗浄した水素化ナトリウム（0.24g，5.90mmol）のN,N－ジメチルホルムアミド懸濁液（15ml）中に1－（4－ニトロベンジル）－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジン（1.60g，5.36mmol）を氷冷下少量ずつ加え、室温にて30分間撹拌した後、ブロモアセトフェノン（1.17g，5.90mmol）のジメチルホルムアミド溶液（3ml）を氷冷下滴下し、更に室温にて3時間撹拌した。反応液を水で希釈し酢酸エチルで抽出した。有機層を飽和食塩水で洗浄し、無水硫酸ナトリウムで乾燥し、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、橙色アモルファス状の標記化合物（1.75g，78%）を得た。

【0155】（B） 1－（4－アミノベンジル）－3－フェナシル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1（F）と同様に、実施例13（A）で得たニトロ体1.68g（4.03mmol）、塩化スズ（II）二水和物3.64g（16.14mmol）、エタノール34mlを用いて、薄茶色アモルファス状の標記化合物（1.53g，98%）を得た。

【0156】（C） 1－{4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル}－3－フェナシル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1（G）と同様に、実施例13（B）で得たアミン体1.59g（4.12mmol）、47%臭化水素酸1.43ml（12.36mmol）、亜硝酸ナトリウム0.31g（4.53mmol）、アセトン10ml、アクリル酸エチル2.68ml（24.72mmol）、酸化銅（I）61mg（0.41mmol）を用いて、茶色油状の標記化合物（2.28g，定量的）を得た。

【0157】（D） 1－{4－[（2,4－ジオキソチアゾリジン－5－イル）メチル]ベンジル}－3－フェナシル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1（H）と同様に、実施例13（C）で得たハロエステル体2.28g（4.12mmol）、チオ尿素310mg（4.12mmol）、酢酸ナトリウム340mg（4.12mmol）、エタノール（20ml）を用いて、薄黄色アモルファス状の標記粗化合物（1.24g，60%）を得た。

【0158】（E） 1－{4－[（2,4－ジオキソチアゾリジン－5－イル）メチル]ベンジル}－3－フェナシル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド[2,3－d]ピリミジンの合成法

実施例1（I）と同様に、実施例12（D）で得たイミン体1.24g（2.48mmol）、2N塩酸12ml、エタノール20mlを用いて、白色アモルファス状の標記化合物（1.13g，91%）を得た。この物のNMRデータを以下に示す。

【0159】^{1}H－NMR（CDCl$_3$，400MHz，TMS） δ：3.08（1H,dd,J=9.9，14.1Hz），3.49（1H,dd,J=3.7，14.1Hz），4.48（1H,dd,J=3.7，9.9Hz），5.54（2H,s），5.57（2H,s），7.15～7.65（9H,m），7.25（1H,dd,J=4.8，7.7Hz），8.48（1H,dd,J=2.0，7.7Hz），8.70（1H,dd,J=2.0，4.8Hz）

【0160】（実施例14）

1－{4－[（2,4－ジオキソチアゾリジン－5－イル）メチル]ベンジル}－3－[2－（ベンジルチオ）エチル]－2,4－ジオキソ－1,2,3,4－テトラヒド

ロピリド［2,3－d］ピリミジン（SSSN）の合成法

（A） 2－アミノ－N－〔2－（ベンジルチオ)エチル〕ニコチンアミドの合成法

1,2－ジヒドロ－2,4－ジオキソピリド［2,3－d］－3,1－オキサジンおよび1,2－ジヒドロ－2,4－ジオキソピリド［3,2－d］－3,1－オキサジンの（4：1）混合物（10.0g，60.93mmol）とベンジルチオエチルアミン塩酸塩（12.41g，60.93mmol）をトルエン（120ml）に懸濁させ、トリエチルアミン（17.88ml，127.95mmol）を加えて一夜還流した。反応液を酢酸エチルで抽出して、有機層を飽和食塩水で洗浄して硫酸マグネシウムで乾燥した後、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製して、茶色固体の標記化合物（13.01g，74%）を得た。

【0161】（B） 3－〔2－（ベンジルチオ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

2－アミノ－3－〔2－（ベンジルチオ）エチル〕ニコチンアミド（13.01g，45.27mmol）および1,1'－カルボニルジイミダゾール（14.68g，90.54mmol）を130℃にて融解させ、さらに30分間撹拌した。反応液を冷却し生じた固体にエタノールを加えて洗浄、濾取、乾燥し、白色粉末状の標記化合物（12.19g，86%）を得た。

【0162】（C） 1－（4－ニトロベンジル）－3－〔2－（ベンジルチオ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

n－ヘキサンで洗浄した水素化ナトリウム（0.92g，22.98mol）のN,N－ジメチルホルムアミド懸濁液（30ml）中に3－〔2－（ベンジルチオ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（6.0g，19.15mmol）を氷冷下少量ずつ加え、室温にて30分間撹拌した。4－ニトロベンジルブロミド（4.55g，21.07mol）を氷冷下少量ずつ加えて、更に室温にて3時間撹拌した。反応液を氷水に注ぎ酢酸エチルで抽出して、飽和食塩水で洗浄した後、硫酸マグネシウムで乾燥して溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、白色アモルファス状の標記化合物（7.88g，92%)を得た。

【0163】（D） 1－（4－アミノベンジル）－3－〔2－（ベンジルチオ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（F）と同様に、実施例14（C）で得たニトロ体7.88g（17.57mmol）、塩化スズ（II）二水和物15.86g（70.29mmol）、エタノール40mlを用いて、薄黄色アモルファス状の標記化合物（5.81g，79%）を得た。

【0164】（E） 1－〔4－（2－ブロモ－2－エトキシカルボニル）ベンジル〕－3－〔2－（ベンジルチオ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（G）と同様に、実施例14（D）で得たアミン体5.81g（13.88mmol）、47%臭化水素酸4.64ml（41.65mmol）、亜硝酸ナトリウム1.05g（15.27mmol）、アセトン20ml、アクリル酸エチル9.03ml（83.30mmol）、酸化銅（I）206mg（1.39mmol）を用いて、白色アモルファス状の標記化合物（5.00g，62%）を得た。

【0165】（F） 1－〔4－〔（2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル）－3－〔2－（ベンジルチオ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（H）と同様に、実施例14（E）で得たハロエステル体5.00g（8.58mmol）、チオ尿素719mg（9.44mmol）、酢酸ナトリウム774mg（9.44mmol）、エタノール40mlを用いて、白色アモルファス状の標記粗化合物（4.56g，定量的）を得た。

【0166】（G） 1－〔4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－〔2－（ベンジルチオ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（I）と同様に、実施例14（F）で得たイミン体4.56g（8.58mmol）、2N塩酸50ml、エタノール100mlを用いて、白色アモルファス状の標記化合物（3.52g，77%）を得た。この物のNMRデータを以下に示す。

【0167】^{1}H－NMR（CDCl$_3$，400MHz，TMS） δ：3.09（1H,dd,J＝9.7，14.1Hz），3.49（1H,dd,J＝3.8，14.1Hz），3.80（2H,t,J＝7.0Hz），4.31（2H,t,J＝7.0Hz），4.48（1H,dd,J＝3.8，9.7Hz），5.54（2H,s），7.15（2H,d,J＝8.0Hz），7.22（1H,dd,J＝4.8，7.7Hz），7.20～7.36（5H,m），7.45（2H,d,J＝8.0Hz），8.47（1H,dd,J＝2.0，7.7Hz），8.67（1H,dd,J＝2.0，4.8Hz）

【0168】（実施例15）

1－〔4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－〔2－（4－ブロモ－2－フルオロベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（SSSO）の合成法

（A） 2－アミノ－N－〔2－（4－ブロモ－2－フ

ルオロベンジルオキシ）エチル〕ニコチンアミドの合成法
実施例14（A）と同様に、実施例1（A）で得たオキサジン2.79g（17.0mmol）、2－（4－ブロモ－2－フルオロベンジルオキシ）エチルアミン8.11g（17.0mmol）、トリエチルアミン2.73ml（19.2mmol）、トルエン50mlを用いて、黄色固体の標記化合物（3.90g，62%）を得た。
〔0169〕（B）　3－〔2－（4－ブロモ－2－フルオロベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法
実施例14（B）と同様に、実施例15（A）で得たピリジン3.90g（10.59mmol）、1,1'－カルボニルジイミダゾール3.43g（21.18mmol）を用いて、薄黄色アモルファス状の標記化合物（3.99g，96%）を得た。
〔0170〕（C）　1－（4－ニトロベンジル）－3－〔2－（4－ブロモ－2－フルオロベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法
実施例14（C）と同様に、実施例15（B）で得たピリミジン3.99g（10.21mmol）、4－ニトロベンジルブロミド2.40g（11.13mmol）、60%水素化ナトリウム0.45g（11.13mmol）、N,N－ジメチルホルムアミド20mlを用いて、薄橙色固体の標記化合物（2.15g，40%）を得た。
〔0171〕（D）　1－（4－アミノベンジル）－3－〔2－（4－ブロモ－2－フルオロベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法
実施例1（F）と同様に、実施例15（C）で得たニトロ体2.15g（4.06mmol）、塩化スズ（II）二水和物3.67g（16.25mmol）、エタノール20mlを用いて、薄黄色粉末状の標記化合物（1.61g，79%）を得た。
〔0172〕（E）　1－〔4－（2－ブロモ－2－エトキシカルボニル）ベンジル〕－3－〔2－（4－ブロモ－2－フルオロベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法
実施例1（G）と同様に、実施例15（D）で得たアミン体1.57g（3.14mmol）、47%臭化水素酸1.05ml（9.42mmol）、亜硝酸ナトリウム0.24g（3.46mmol）、アセトン5ml、アクリル酸エチル2.04ml（18.84mmol）、酸化銅（I）46mg（0.31mmol）を用いて、黄色油状の標記化合物（0.80g，44%）を得た。
〔0173〕（F）　1－〔4－〔（2－イミノ－4－オキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－〔2－（4－ブロモ－2－フルオロベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法
実施例1（H）と同様に、実施例15（E）で得たハロエステル体0.80g（1.37mmol）、チオ尿素115mg（1.51mmol）、酢酸ナトリウム124mg（1.51mmol）、エタノール5mlを用いて、白色アモルファス状の標記粗化合物（0.84g，定量的）を得た。
〔0174〕（G）　1－〔4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－〔2－（4－ブロモ－2－フルオロベンジルオキシ）エチル〕－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法
実施例1（I）と同様に、実施例15（F）で得たイミン体0.84g（1.37mmol）、2N塩酸15ml、エタノール30mlを用いて、白色アモルファス状の標記化合物（0.50g，60%）を得た。この物のNMRデータを以下に示す。
〔0175〕^{1}H－NMR（CDCl$_3$，400MHz，TMS）　δ：3.10（1H,dd,J＝9.9，14.1Hz），3.49（1H,dd,J＝3.8，14.1Hz），3.82（2H,t,J＝5.7Hz），4.38（2H,t,J＝5.7Hz），4.49（1H,dd,J＝3.8，9.9Hz），4.55（2H,s），5.53（2H,s），7.13（2H,d,J＝8.0Hz），7.18～7.26（4H,m），7.45（2H,d,J＝8.0Hz），8.45（1H,dd,J＝2.0，7.7Hz），8.68（1H,dd,J＝2.0，4.8Hz）
〔0176〕（実施例16）
1－〔4－〔（2,4－ジオキソチアゾリジン－5－イル）メチル〕ベンジル〕－3－イソプロピル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジン（SSSP）の合成法
（A）　2－アミノ－N－イソプロピルニコチンアミドの合成法
1,2－ジヒドロ－2,4－ジオキソピリド［2,3－d］－3,1－オキサジンおよび1,2－ジヒドロ－2,4－ジオキソピリド［3,2－d］－3,1－オキサジンの（4：1）混合物（7.0g，42.65mmol）とイソプロピルアミン（11.04ml，127.96mmol）をN,N－ジメチルホルムアミド（50ml）に懸濁させ、氷冷下、2時間撹拌した。反応液は酢酸エチルで抽出し、有機層を飽和食塩水で洗浄し、無水硫酸ナトリウムで乾燥し、溶媒を減圧留去した。残渣をシリカゲルカラムクロマトグラフィーで精製し、薄黄色固体の標記化合物（1.74g，23%）を得た。
〔0177〕（B）　3－イソプロピル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法
実施例14（B）と同様に、実施例16（A）で得たピ

リジン2.25g（12.55mmol）、1,1'-カルボニルジイミダゾール4.07g（25.11mmol）を用いて、白色粉末状の標記化合物（2.29g，89%）を得た。

【0178】（C） 1－（4－ニトロベンジル）－3－イソプロピル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例14（C）と同様に、実施例16（B）で得たピリミジン2.19g（10.67mmol）、4－ニトロベンジルブロミド2.31g（10.67mmol）、60%水素化ナトリウム0.47g（11.74mmol）、N,N－ジメチルホルムアミド25mlを用いて、黄色アモルファス状の標記化合物（3.47g，96%）を得た。

【0179】（D） 1－（4－アミノベンジル）－3－イソプロピル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（F）と同様に、実施例16（C）で得たニトロ体3.41g（10.02mmol）、塩化スズ（II）二水和物9.04g（40.08mmol）、エタノール20mlを用いて、薄黄色非晶状の標記化合物（3.17g，定量的）を得た。

【0180】（E） 1－｛4－（2－ブロモ－2－エトキシカルボニルエチル）ベンジル｝－3－イソプロピル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（G）と同様に、実施例16（D）で得たアミン体3.17g（10.21mmol）、47%臭化水素酸3.53ml（30.63mmol）、亜硝酸ナトリウム0.78g（11.24mmol）、アセトン20ml、アクリル酸エチル6.64ml（61.26mmol）、酸化銅（I）15mg（1.02mmol）を用いて、薄黄色油状の標記化合物（1.79g，37%）を得た。この物のNMRデータを以下に示す。

【0181】^{1}H－NMR（CDCl$_3$，400MHz，TMS） δ：1.20（3H,t,J=7.1Hz），1.52（3H,s），1.54（3H,s），3.19（1H,dd,J=7.0，14.3Hz），3.41（1H,dd,J=8.4，14.3Hz），4.15（2H,q,J=7.1Hz），4.34（1H,dd,J=7.0，8.4Hz），5.31（1H,m），5.51（2H,s），7.14（2H,d,J=8.2Hz），7.18（1H,dd,J=4.8，7.7Hz），7.42（2H,d,J=8.2Hz），8.44（1H,dd,J=2.0，7.7Hz），8.62（1H,dd,J=2.0，4.8Hz）

【0182】（F） 1－｛4－｛（2－イミノ－4－＊オキソチアゾリジン－5－イル）メチル｝ベンジル｝－3－イソプロピル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（H）と同様に、実施例16（E）で得たハロエステル体2.89g（6.09mmol）、チオ尿素560mg（7.31mmol）、酢酸ナトリウム600mg（7.31mmol）、エタノール25mlを用いて、白色アモルファス状の標記粗化合物（1.90g，74%）を得た。

【0183】（G） 1－｛4－｛（2,4－ジオキソチアゾリジン－5－イル）メチル｝ベンジル｝－3－イソプロピル－2,4－ジオキソ－1,2,3,4－テトラヒドロピリド［2,3－d］ピリミジンの合成法

実施例1（I）と同様に、実施例16（F）で得たイミン体1.88g（4.44mmol）、2N塩酸22ml、エタノール30mlを用いて、白色アモルファス状の標記化合物（1.83g，97%）を得た。この物のNMRデータを以下に示す。

【0184】^{1}H－NMR（CDCl$_3$，400MHz，TMS） δ：1.52（3H,s），1.54（3H,s），3.10（1H,dd,J=9.9，14.1Hz），3.50（1H,dd,J=4.0，14.1Hz），4.49（1H,dd,J=4.0，9.9Hz），5.32（1H,m），5.52（2H,s），7.16（2H,d,J=8.1Hz），7.19（1H,dd,J=4.8，7.9Hz），7.45（2H,d,J=8.1Hz），8.45（1H,dd,J=2.0，7.9Hz），8.63（1H,dd,J=2.0，4.8Hz）

【0185】（試験例）次に、一般式（I）で表される本発明の化合物の有効性を示す薬理試験の結果について説明するが、ここに例示しない本発明の化合物についても同様の効果が認められた。

［1］血糖降下作用

実験には、13～20週齢のKK/Ta Jcl雄性マウスを1群4～5匹として用いた。試験開始3日前に眼窩静脈叢よりヘパリン採血（30μl）し、グルコースオキシダーゼ法にて血糖値を求めた。血糖値が350～400mg／dl以上の個体を選び、各群の平均血糖値が等しくなるように群分けした。各マウスは個別ゲージに入れ、薬物を100mg／kgの用量で1日2回経口投与した。4日目投与3時間後に採血を行い、溶媒対照群の血糖値を100として、これに対する血糖降下率（下記数1に示す）を求めた。結果を表1に示す。

【0186】

【数1】

$$\text{血糖降下率（\%）} = \frac{\text{非処置糖尿病群の血糖（平均値）} - \text{検体または対照薬剤投与群の血糖値（平均値）}}{\text{非処置糖尿病群の血糖値（平均値）}} \times 100$$

（数1）

【0187】［2］アルドースリダクターゼ阻害活性（ARI活性）

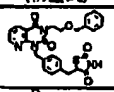
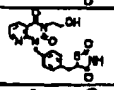
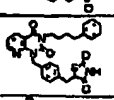
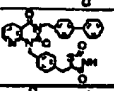
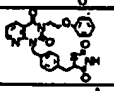
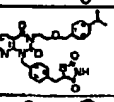
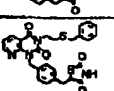
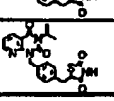
5週齢のWistar系雄性ラットをエーテル麻酔下に致死し、直ちに水晶体を摘出した。水晶体からハイマン（Hayman）らの方法［ジャーナル オブ バイオロジカル ケミストリー（J.Biol.Chem.）,240,877-882（1965）］に準じアルドースリダクターゼを調製した。アルドースリダクターゼ活性の測定はデュフラン（Dufrane）らの方法［バイオケミカル メディシン（Biochem.med.）,32,99-105（1984）］により行った。即ち100mM硫酸リチウム、0.03mM NADPH（還元型ニコチンアミド アデニンジヌクレオチド ホスフェート）および基質として0.1mM DL－グリセロアルデヒドを含むように調製した135mMナトリウム－カリウム－リン酸緩衝液（pH7.0）400μlに上記アルドースリダクターゼ50μlを加え、30℃にて30分間反応させた。

【0188】次に、0.5N塩酸0.15mlを加えて反応を停止させ、10mMイミダゾールを含む6N水酸化ナトリウム0.5mlを添加することにより、前記反応によって生じたNADP（酸化型ニコチンアミド アデニン ジヌクレオチド ホスフェート）を蛍光物質に変換して、60分後にその蛍光強度を測定した。蛍光強度は、室温でMTP－100Fコロナマイクロプレートリーダ（コロナ電気株式会社）を用いて励起波長360nm、蛍光波長460nmの条件で測定した。また、検体を加える代わりにエタノールを加える以外は上記と同様に反応させて測定した蛍光強度をコントロール値とした。検体のアルドースリダクターゼ阻害活性は蛍光強度の減少よりアルドースリダクターゼ阻害活性を50%阻害するのに必要な濃度（50%阻害濃度；IC_{50}）として求めた。アルドースリダクターゼ阻害活性の結果を表1に示す。

【0189】

【表1】　＊

＊　表1　血糖降下率およびARI活性

番号	被検薬物（構造式）	血糖降下率（%）	ARI活性 IC_{50} (M)
(XXII)		59	1.48×10^{-6}
(XXIII)		51	5.75×10^{-6}
(XXIV)		53	1.32×10^{-6}
(XXV)		51	$>10^{-5}$
(XXVI)		49	9.32×10^{-7}
(XXX)		60	7.35×10^{-6}
(XXXVI)		45	4.92×10^{-7}
(XXXVII)		48	6.30×10^{-6}

【0190】（急性毒性）ICR系雄性マウス（5週齢）を用いて、経口投与による急性毒性試験を行った。本発明のピリドピリミジン誘導体のLD_{50}値はいずれも300mg／kg以上であり、高い安全性が確認された。

【0191】

【発明の効果】本発明の一般式（I）で表わされる新規なピリドピリミジン誘導体は、血糖降下作用およびAR阻害作用を併せ持ち、それを含有する医薬組成物は、例えば白内障、神経症、網膜症、腎障害等の糖尿病における各種合併症の予防および治療的処置のための薬剤として有効である。

フロントページの続き

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A61K 31/505	AED		A61K 31/505	AED	

MULTIVALENT COMPOUNDS FOR CROSSLINKING RECEPTORS AND USES THEREOF

Cross Reference To Related Applications

This application is a continuation-in-part of Application Serial No. 08/837,305, filed April 11, 1997, which is a continuation-in-part of Application Serial No. 08/671,756, filed June 28, 1996.

Statement as to Federally Sponsored Research

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The Field of the Invention

This invention relates to low molecular weight bivalent and multivalent crosslinking compounds capable of inducing association between naturally occurring receptors, and particularly to inducing association between surface receptors on T cells, hematopoietic cells or antigen presenting cells such as macrophages, dendritic cells or B cells, e.g., homobivalent induced CD26-CD26 association or heterobivalent induced CD26 association with the above-mentioned surface receptors.

Background of the Invention

Cell surface receptors transmit signals received on the outside of a cell to the inside through two basic mechanisms: (1) ligand-induced allosteric conformational change and (2) ligand-induced association.

The ligands for the ligand-induced, allosteric conformational change mechanism are typically small molecules, such as the catecholamines or the neuropeptide hormones.

The ligand-induced association mechanism involves an association of specific proteins on the cell surface and has only recently been discovered (relatively speaking), but already has been shown to be as widely used and as important as the first mechanism.

Receptors activated by a ligand-induced dimerization include, for example, those for cell growth and differentiation factors. Factors which serve as ligands for these receptors are typically large polypeptide hormone and cytokines such as erythropoietin, granulocyte colony stimulating factor (G-CSF), or granulocyte macrophage colony stimulating factor (GM-CSF), and human growth hormone (hGH). Many of the dimerization-activated receptors have cytoplasmic tails that contain protein kinase domains or docking sites. Ligand-induced dimerization of the extracellular domains of these receptors results in the juxtaposition of their cytoplasmic tails. They then presumably phosphorylate each other in trans and thereby initiate the cytosolic signaling pathway. In some cases the cytoplasmic domains of dimerization-activated receptors do not have kinase domains themselves, but function the same as if they did because they associate with protein kinases via docking sites.

Receptors activated by oligomerization or aggregation are found most frequently in the immune system. They include, for example, the T cell surface receptors such as CD2, CD4, CD8, CD28, CD26, CD44, CD45, CD10, and CD3/TCR (T cell antigen receptor) and the B cell surface receptors such as CD40, B7.1 and B7.2. The ligands for these cell receptors are most often cell surface proteins themselves, and can be found on cognate cells. Aggregation-activated receptors frequently have short cytoplasmic domains which act to bind and thereby recruit other cell surface and/or cytosolic factors following the aggregation of their extracellular domains.

The allosterically activated receptor class has been the primary focus of drug discovery, design, and development efforts for decades. These efforts have yielded many pharmacologically agents. In principle, two distinct types of agents are possible: antagonists and agonists. Antagonists block the binding of the natural ligand without inducing the conformational change in the receptor thereby blocking a signal transduction pathway. Agonists bind to the receptor in a manner which mimics the natural ligand closely enough to induce the same conformational change

as natural ligand thereby initiating a signal transduction pathway. See Seed, et al. for a theoretical discussion on how to make an agonist from an antagonist (Seed, B., Making Agonists of Antagonists, Chemistry & Biology 1:125 (1994). See Austin, et al. for a discussion of the role of regulated protein dimerization in biology (Austin, et al. Chemistry & Biology 1:131 (1994)).

Several association-activation receptors have recently become the targets of drug discovery efforts, owing to the important roles they play in various cellular signaling. Low-molecular weight synthetic molecules, that block the interaction of receptors and their ligands and interfere with signal transduction (i.e., antagonists), have been identified using the methods employed with the allosterically activated class. These low-molecular weight synthetic molecules are potential drugs.

Monomeric inhibitors block recall antigen-induced T cell activation and proliferation (G.R. Flentke, et al. Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhibitors to examine the role of DP-IV in T-cell function, *PNAS (USA)* 88, 1556-1559 (1991)). A number of anti-CD26 mAbs have the same inhibitory activity when used under non-crosslinking conditions (C. Morimoto, et al. 1F7, a novel cell surface molecule, involved in helper function of CD4 cells, *Journal of Immunology* 143, 3430-3439 (1989) and published erratum appears in *J. Immunology.* 144(5):2027 (Mar 1990)). Most anti-CD26 mAbs are stimulatory, rather than inhibitory when used under crosslinking conditions (R.W. Barton, et al. Binding of the T cell activation monoclonal antibody Ta1 to dipeptidyl peptidase IV, *Journal of Leukocyte Biology* 48, 291-296 (1990); L.A. Bristol, et al. Thymocyte costimulating antigen is CD26 (dipeptidyl-peptidase IV). Co-stimulation of granulocyte, macrophage, and T lineage cell proliferation via CD26, *Journal of Immunology* 149, 367-372. (1992); L.A. Bristol, et al. Characterization of a novel rat thymocyte costimulating antigen by the monoclonal antibody 1.3, *Journal of Immunology* 148, 332-338 (1992); B. Fleischer, et al. Triggering of cytotoxic T lymphocytes and NK cells via the Tp103 pathway is dependent on the expression of the T cell receptor/CD3 complex,

Journal of Immunology 141, 1103-11077 (1988); M. Hegen, et al. The T cell triggering molecule Tp103 is associated with dipeptidyl aminopeptidase IV activity, *J. Immunol.* 144, 2980-2914 (1990)).

A class of low molecular weight synthetic monomeric molecules with high affinity for CD26 have previously been developed and characterized (G.R. Flentke, et al. Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhibitors to examine the role of DP-IV in T-cell function, *PNAS (USA)* 88, 1556-1559 (1991); W.G. Gutheil and W.W. Bachovchin. Separation of L-Pro-DL-boroPro into Its Component Diastereomers and Kinetic Analysis of Their Inhibition of Dipeptidyl Peptidase IV. A New Method for the Analysis of Slow, Tight-Binding Inhibition, *Biochemistry* 32, 8723-8731 (1993)). These molecules have been shown to be potent and specific synthetic inhibitors for CD26's associated DP IV proteinase activity. DP-IV is a postproline cleaving enzyme with a specificity for removing Xaa-Pro (where Xaa represents any amino acid) dipeptides from the amino terminus of polypeptides.

Representative monomeric structures of these transition-state-analog-based inhibitors, Xaa-boroPro, are e.g., Pro-boroPro and Ala-boroPro. BoroPro refers to the analog of proline in which the carboxylate group (COOH) is replaced with a boronyl group [$B(OH)_2$]. Pro-boroPro, the most thoroughly characterized of these inhibitors has a Ki of 16 picomolar (pM) (W.G. Gutheil and W.W. Bachovchin. Separation of L-Pro-DL-boroPro into Its Component Diastereomers and Kinetic Analysis of Their Inhibition of Dipeptidyl Peptidase IV. A New Method for the Analysis of Slow, Tight-Binding Inhibition, *Biochemistry* 32, 8723-8731 (1993)). Val-boroPro has even a higher affinity, with a Ki of 1.6 pM (W.G. Gutheil and W.W. Bachovchin. Supra; R.J. Snow, et al. Studies on Proline boronic Acid Dipeptide Inhibitors of Dipeptidyl Peptidase IV: Identification of a Cyclic Species Containing a B-N Bond, *J. Am. Chem. Soc.* 116, 10860-10869 (1994)). Thus, these Xaa-boroPro inhibitors are about 10^{+6} fold more potent than the next best known inhibitors. In comparison, antibodies usually have affinities for their

targets between 10^{-8} and 10^{-9} M.

United States Patent Nos. 4,935,493 (the '493 patent) and 5,462,928 (the '928 patent), both of which are incorporated herein by reference, disclose protease inhibitors and transition state analogs (the '493 patent) and methods for treating transplant rejection in a patient, arthritis, or systemic lupus erythematosis (SLE) by administering a potent inhibitor of the catalytic activity of soluble amino peptidase activity of dipeptidyl peptidase type IV (DP-IV; (G.R. Flentke, et al. Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhibitors to examine the role of DP-IV in T-cell function, *PNAS (USA)* 88, 1556-1559 (1991)).

Until now, most drug discovery and development efforts have been directed at the allosteric conformational change-activated class of receptors. Also, the efforts directed at the association-activated class have focused on monomeric agents capable of blocking binding of a natural ligand, and therefore blocking signal transduction mediated by these receptors.

Cytotoxic drugs have untoward effects since they indiscriminately kill all proliferating cells. With the advent of monoclonal antibodies, it is possible to increase the specificity of these therapeutic tools. Monoclonal antibodies against the T cell receptors, e.g., T-cell receptor, CD4 and CD8 co-receptors, and to MHC class II molecules, have all been evaluated for their respective benefit in experimental models for the treatment of autoimmune disease. The major impediment to using monoclonal antibodies as a therapeutic tool in humans, is that most monoclonal antibodies are made in mice, and humans rapidly develop an antibody response to mouse antibodies, which limits their potency because of neutralization and, worse, produces allergic reactions such as immune complex disease. Once this has occurred, all mouse monoclonal antibodies become useless in that patient. To avoid this problem, antibodies which are not recognized as foreign by the human immune system are currently being made via different ways.

One approach is to clone human V regions into a phage display library and select for binding to human cells. Using this method, monoclonal antibodies that are entirely human in origin can be obtained. Second, mice that lack endogenous immunoglobulin genes can be made transgenic for human heavy and light chain loci using yeast artificial chromosomes. Third, one may graft the antigen-binding loops of a mouse monoclonal antibody onto the framework of a human immunoglobulin molecule (a process known as humanization).

Each of these methods produce monoclonal antibodies which are far less immunogenic in humans than the parent mouse monoclonal antibodies, but with each methods comes a host of additional problems or road-blocks. For example, antiidiotypic neutralizing antibodies are often produced in patients receiving monoclonal antibody therapy.

Summary of the Invention

In general, low molecular weight, bivalent or multivalent, synthetic crosslinking compounds are designed and developed. These synthetic crosslinking compounds may act either as agonists or antagonists and induce association between naturally occurring receptors, e.g., induce the association of one particular T cell surface receptor, such as CD26, with (a) itself or, (b) with another T cell or antigen presenting cell surface receptor (e.g., CD2, CD4, CD8, CD28, CD26, CD44, CD45, CD10, CD3/TCR (or TCR/CD3)), CD40, B7.1 and B7.2.

The low molecular weight, bivalent or multivalent, synthetic crosslinking compounds of this invention are small enough (less than about 30 amino acids, and more preferably about 20 amino acids) to obviate the immunogenicity associated with monoclonal antibodies.

The bivalent or multivalent, synthetic crosslinking compounds of this invention can be administered to a patient without being co-administered with an adjuvant. In contrast, most other peptides, proteins and carbohydrate antigens are usually poorly immunogenic, or not immunogenic at all, when administered without

adjuvant.

The compounds of the invention are useful for crosslinking molecules on the same or different cells that are involved in immune system modulation. Compounds of the invention fall within the following genus:

(I) $[P^2(R^2)_m]_n$-L-P^1R^1]

wherein P^1 represents a first targeting moiety, preferably a peptide that can mimic the substrate binding site of a protease (preferably a serine protease or cysteine protease) that is expressed on the surface of the cell involved in immune system modulation (e.g., a T cell, a B cell, a stem cell, a bone marrow cell, including an antigen presenting cell);

R^1 represents a reactive group that reacts with a functional group in a reactive center of the protease;

P^2 represents a second targeting moiety, preferably a peptide, that may be the same or different from the first targeting moiety;

R^2 represents a second reactive group that may be the same or different from the first reactive group;

m = 0 or 1 and n is a whole number from 1 to 10, and

L represents a linker molecule (i) having a molecular weight ranging from about 100 daltons to about 2000 daltons, (ii) having a length ranging from about 20Å to about 300 Å; and (iii) containing a chain of atoms selected from the group consisting of C, O, N, S, and phosphorus atoms, connected by single or by double bands. Thus, P^1 can be D1~A1~A2~A3~A4 or D2~A5~A6~A7~A8 as described below in reference to certain embodiments of the invention. In important embodiments of the invention, P^1 is a peptide or a peptidomimetic.

In certain embodiments of the invention, if P^2=P^1, then R^2 can be absent, the same, or different from R^1. In general, n is 1 and the compounds of the

invention are referred to as homodimers (i.e., $P^2=P^1$) or heterodimers (i.e., $P^2 \neq P^1$).

Cells which are involved in immune system modulation are blood cells including T cells, B cells, stem cells, bone marrow cells, dendritic cells, and other antigen presenting cells. The P^1 targeting moiety can have a carboxyl terminal portion containing 1, 2, 3, or 4 amino acids which mimic the substrate binding site of the protease. Exemplary proteases which are believed to be expressed on the surface of such cells and which are bound by the P^1 targeting moieties include post-prolyl cleaving enzymes, trypsin, chymotrypsin and elastase. The particular amino acids in the naturally occurring substrates of these enzymes are well known in the art and are identified below.

In general, the compounds of the invention contain from 1 to 30 amino acid residues (preferably the L isomers) and, more preferably, contain from 1 to 20 amino acid residues. In the most preferred embodiments, the P^1 targeting moieties contain from 1 to 10 amino acids, most preferably, from 1 to 2 amino acids. The P^1 targeting moiety can contain L or D amino acids; however, it is preferred that at least the amino acids which mimic the substrate binding site be in the L configuration. In contrast, the amino acids which are in a retroinverso configuration (see the Examples) preferably are in the D configuration.

The composition of the P^1 targeting moiety is not limited to amino acids but may include, in whole or in part, non-amino acid components, provided that such components do not interfere significantly (i.e., do not lower the Ki of the compound to less than about 10^{-7}M with the site-specific recognition of the compound by the protease and provided that the non-amino acid components do not interfere with the formation of a complex between the compound and the protease. In certain embodiments, the portion of the P^1 targeting moiety that is involved in binding to the substrate binding site is formed of amino acids and the remaining portion of the P^1 targeting moiety is formed of non-amino acid components. In general, any portion of the P^1 targeting moiety can be modified, for example, to be

coupled to a detectable reagent, or immobilized to a surface via a linker, provided that such modifications satisfy the foregoing inhibition constant and complex formation criteria.

Peptides which reportedly have utility for inhibiting post-prolyl cleaving enzymes and which, if coupled to a reactive group, form a covalent complex with a functional group in the reactive site of a post-prolyl cleaving enzyme are described in U.S. Patent No. 4,935,493, "Protease Inhibitors", issued to Bachovchin et al. ("Bachovchin '493"); U.S. 5,462,928, "Inhibitors of Dipeptidyl-aminopeptidase Type IV", issued to Bachovchin et al. ("Bachovchin '928"); U.S. 5,543,396, "Proline Phosphonate Derivatives", issued to Powers et al., ("Powers '396"); U.S. 5,296,604, "Proline Derivatives and Compositions for Their Use as Inhibitors of HIV Protease", issued to Hanko et al., ("Hanko '604"); PCT/US92/09845, "Method for Making a Prolineboronate Ester", and its U.S. priority applications (USSN 07/796,148 and 07/936,198), Applicant Boehringer Ingelheim Pharmaceuticals, Inc. ("Boehringer"); and PCT/GB94/02615, "DP-IV-Serine Protease Inhibitors", Applicant Ferring V.V. ("Ferring").

In important embodiments, the P^1 targeting moiety mimics the substrate binding site of the post-prolyl cleaving enzyme DP IV (also referred to herein as "CD 26"). DP IV is a post-prolyl cleaving enzyme with a specificity for removing Xaa-Pro (where Xaa represents any amino acid) dipeptides from the amino terminus of a polypeptide substrate. Representative structures of transition-state analog-based inhibitors Xaa-boroPro, include Lys-BoroPro, Pro-BoroPro and Ala-BoroPro in which "boroPro" refers to the analog of proline in which the carboxylate group (COOH) is replaced with a boronyl group [$B(OH)_2$]. Alternative crosslinking compounds of the invention have an analogous structure in which the boronyl group is replaced by a phosphonate or a fluoroalkylketone (described below).

The invention also embraces compounds which mimic the substrate binding site of other post-prolyl cleaving enzymes. For example, IgA 1 proteases

recognize the cleavage site Ser-Thr-Pro-Pro-X (where X is any amino acid). Accordingly, Ser-Thr-Pro-Pro-R^1 is suitable for selectively binding to, and forming a complex with a functional group in the active site of an IgA 1 protease. The Ser-Thr in this targeting moiety may be readily substituted with any of the 20 naturally occurring amino acids, most preferably those having non-bulky side groups, such as Ala and Gly. It also is possible to substitute non-naturally occurring amino acids, such as 2-azetidinecarboxylic acid or pipecolic acid (which have 6-membered, and 4-membered ring structures respectively) for either of the Pro residues. Those skilled in the art will recognize that there are other such changes which can be made without significantly affecting the binding and complex forming character of these compounds.

In the case of IgA 2 protease, the cleavage site in the natural substrate is Pro-Thr-Pro-X with hydrolysis occurring between Pro and X. Thus, a preferred P^1 R^1 binding moiety for binding to an IgA 2 protease has the formula Pro-Thr-Pro-R^1. Thr can be substituted by any of the naturally occurring amino acids, especially ones having non-bulky side groups, such as Ala, Gly or Ser. Other examples of post-prolyl cleaving enzymes which can be targeted by the targeting moieties of the invention include other IgA enzymes, encephalon degrading enzymes, vasopressin degrading enzymes, and oxytocin degrading enzymes.

The P^1 targeting moieties of the invention can be designed to mimic the substrate binding sites of other, non-post-prolyl cleaving enzymes that may be expressed on the surface of cells involved in immune system modulation. These enzymes include, for example, cysteine proteases and serine proteases such as trypsin, chymotrypsin and elastase. The substrate binding sites for these enzymes are well known and peptidomimetics for targeting binding to these sites have been reported. For example, a P^1 targeting moiety of the invention which mimics the substrate binding site of trypsin would include an arginine (Arg) or lysine (Lys) residue at its carboxyl-terminus with the carboxyl group of the Arg or Lys coupled

to an appropriate reactive center, R^1, to form a covalent bond with a functional group in the active site of trypsin. Exemplary borolysine targeting moieties that can be used to form the compounds of the invention are described in U.S. Patent Nos. 5,187,157 and 5,242,904, "Peptide Boronic Acid Inhibitors of Trypsin-like Proteases", issued to Kettner et al. ("Kettner '157" and Kettner '5,242,904) and in U.S. Patent No. 5,288,707, "Borolysine Peptidomimetics", issued to Metternich ("Metternich"). Intermediates that are useful for preparing these inhibitors and related procedures are described in U.S. 5,250,720, "Intermediates for Preparing Peptide Boronic Acid Inhibitors of Trypsin-like Proteases" ("Kettner '720") and U.S. 5,384,410, "Removal of Boronic Acid Protecting Groups by Transesterification" ("Kettner '410").

P^1 targeting moieties also can be designed to mimic the substrate binding site of chymotrypsin. Such targeting moieties include a carboxyl terminal amino acid residue that is selected from the group consisting of phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr). Preferably, the carboxyl groups of these amino acids are covalently coupled to an R^1 reactive group to form a binding moiety that selectively binds to, and forms a covalent complex with, a functional group in the active site of chymotrypsin. Yet other P^1 targeting moieties of the invention can be designed which mimic the substrate binding site of an elastase. For example, a P^1 targeting moiety which mimics the substrate binding site of an elastase would include a carboxyl terminal amino acid residue that is alanine (Ala) or glycine (Gly) with the carboxyl group of these amino acids covalently coupled to the reactive group R^1. In general, conventional chemical reactions can be used to form the foregoing P^1R^1 binding moieties. Thus, P^1R^1 binding moieties of the invention can be designed and constructed to mimic the substrate binding site of virtually any protease for which the natural substrate is known or can be identified.

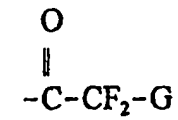
The development of phage display libraries and chemical combinatorial libraries from which synthetic compounds can be selected which mimic the

substrate binding site of a protease permits the identification of further P^1 targeting moieties to which an R^1 reactive group can be covalently attached to form a binding moiety which mimics the substrate binding site of the protease and which forms a complex with a functional group in the protease reactive site. Such libraries can be screened to identify non-naturally occurring putative targeting moieties by assaying protease cleavage activity in the presence and absence of the putative phage display library molecule or combinatorial library molecule and determining whether the molecule inhibits cleavage by the protease of its natural substrate or of a substrate analog (e.g., a chromophoric substrate analog which is easily detectable in a spectrophotometric assay). Those phage library and/or combinatorial library molecules which exhibit inhibition of the protease then can be covalently coupled to the reactive groups R^1 disclosed herein and again tested to determine whether these novel molecules selectively bind to the protease (e.g., by repeating the above-noted screening assay). In this manner, a simple, high-through-put screening assay is provided for identifying non-naturally occurring targeting moieties of the invention.

In general, the first targeting moieties of the invention are covalently coupled via a carboxyl group at their carboxyl terminal amino acid to a first reactive group, R^1. As used herein, R^1 refers to a reactive group that is capable of reacting with a functional group in a reactive center of a protease expressed on the surface of a cell involved in immune system modulation. By reacting with a reactive center of the target protease, it is meant that the R^1 forms a covalent bond with a functional group that is located in the active site. R^1 reactive groups that are embraced within the invention include the reactive groups referred to as group "T" in US 4,935,493, "Protease Inhibitors", issued to Bachovchin, et al. These include boronate groups, phosphonate groups, and fluoroalkylketone groups. The boronate groups are described in the detailed description of the invention and in the Examples. The phosphonate and fluoroalkylketone groups are described below. In general, it is preferred that the linkage between the carboxyl terminus of the targeting moiety and

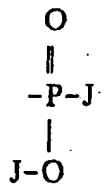
the reactive group be in an L configuration. It is preferred that the reactive group forms a covalent band with a functional group in the active site; however, there is no requirement for covalent bond formation in order to form a complex between the binding moiety and the active site.

The reactive groups of the invention that are phosphonate groups have the formula:



where G is either H, F or an alkyl group containing 1 to about 20 carbon atoms and optional heteroatoms which can be N, S, or O. Additional exemplary proline phosphonate derivatives which contain a perfluoroalkyl group, a phenyl group or a substituted phenyl group and which can be used in accordance with the methods of the invention are those described in U. S. 5,543,396 (Powers '396).

As used herein, the reactive groups of the invention that are fluoroalkylketone reactive groups have the formula:



where each J, independently, is O-alkyl, N-alkyl, or alkyl (each containing about 1-20 carbon atoms) and, optionally, heteroatoms which can be n=N, S, or O. Other ketoamides, ketoacids and ketoesters that are useful reactive groups for reacting with the reactive center of a protease (e.g., a serine protease or a cysteine protease) are described in PCT/US91/09801, "Peptides, Ketoamides, Ketoacids, and Ketoesters", Applicant: Georgia Tech Research Corp. ("GA Tech") which claims priority to U.S. 635,287, filed Dec. 28, 1990.

In certain embodiments, the reactive groups are selected from the groups having the formulas,

- C - C - NH_2, an alphaketo amide;

- C - C - OR, where R is an alkyl, or aryl group and may be substituted or unsubstituted, an alphaketo ester; and

- C - C - OH, an alphaketo acid.

The reactive groups of the invention also include the reactive groups described in PCT/GB94/02615, "DP-IV-Serine Protease Inhibitors" (Ferring). These include the above-noted boronyl groups [$B(OH)_2$], as well as pyrrolidides and the following reactive groups, any of which may be substituted or unsubstituted provided that the substitution does not adversely affect the functional activity of the reactive group or the binding moiety to which it is attached: CN, C≡C, CHO and CH=NPh, wherein Ph refers to phenyl. These examples are illustrative only and are not intended to limit the scope of the invention. As noted in Ferring, compounds containing these representative reactive groups can be prepared by an adaptation of the general route described by E. Schon et al., Biol, Chem. Hoppe-Seyler:372:305-311 (1991) and by W.W. Bachovchin et al., J. Biol. Chem. 265:3738-3743 (1990). (See, also, the above-referenced Bachovchin United States patents.

The second targeting moiety, P^2, binds to a molecule that is present on the surface of the same or different cell to which the first targeting moiety binds. Preferably, the second targeting moiety binds to a molecule (e.g., a receptor, a major histocompatibility complex (MHC) molecule) which is present on the surface of a T cell or on the surface of a B cell. In certain embodiments, the second targeting moiety has a structure which mimics the substrate binding site of a protease that is present on a cell that is involved in immune system modulation.

Thus, the second targeting moiety may be the same as the first targeting moiety, and the compounds of the invention are useful for crosslinking proteases that have the same or a similar substrate specificity on the same or different cells. For example, the compounds of the invention can be used to crosslink a first protease (e.g., a post-prolyl cleaving enzyme) on a first cell and a different protease (e.g., a trypsin, chymotrypsin, elastase or other serine protease or cysteine protease) that is expressed on the surface of the same or on a different second cell. In certain preferred embodiments, the first and second targeting moieties are identical (i.e., $P^2=P^1$) and the second reactive group R^2 may be absent (i.e., m=0), the same or different from the first reactive group R^1 (i.e., $R^1 \neq R^2$). Compounds which include identical P^1 and P^2 groups and identical R^1 and R^1 groups are referred to as "homodimers". In yet other embodiments, the first and second targeting moieties are different and these compounds are referred to as "heterodimers".

In yet other embodiments, the second targeting moiety is an antigen that selectively binds to an MHC molecule on the surface of an antigen presenting cell. Such embodiments of the invention are useful as vaccines for inducing an immune system response to the antigen. In particular, such compounds are useful for inducing an immune system response to antigens that exhibit relatively low immunogenicity using conventional vaccine preparations. Accordingly, the invention also provides for an improved vaccine and related methods for inducing an immune response to an antigen. Examples of antigens are antigens characteristic of pathogens, cancer antigens, and allergens.

Antigens that are characteristic of autoimmune disease typically will be derived from the cell surface, cytoplasm, nucleus, mitochondria and the like of mammalian tissues. Examples include antigens characteristic of uveitis (e.g. S antigen), diabetes mellitus, multiple sclerosis, systemic lupus erythematosus, Hashimoto's thyroiditis, myasthenia gravis, primary myxoedema, thyrotoxicosis, rheumatoid arthritis, pernicious anemia, Addison's disease, scleroderma,

autoimmune atrophic gastritis, premature menopause (few cases), male infertility (few cases), juvenile diabetes, Goodpasture's syndrome, pemphigus vulgaris, pemphigoid, sympathetic ophthalmia, phacogenic uveitis, autoimmune haemolytic anemia, idiopathic thrombocytopenic purpura, idiopathic leucopenia, primary biliary cirrhosis (few cases), ulcerative colitis, Sjogren's syndrome, Wegener's granulomatosis, poly/dermatomyositis, and discoid lupus erythromatosus. Additional examples are provided below.

Antigens that are allergens are generally proteins or glycoproteins, although allergens may also be low molecular weight allergenic haptens that induce allergy after covalently combining with a protein carrier (Remington's Pharmaceutical Sciences). Allergens include antigens derived from pollens, dust, molds, spores, dander, insects and foods. Specific examples include the urushiols (pentadecylcatechol or heptadecylcatechol) of *Toxicodendron* species such as poison ivy, poison oak and poison sumac, and the sesquiterpenoid lactones of ragweed and related plants. Additional examples are provided below.

Antigens that are characteristic of tumor antigens typically will be derived from the cell surface, cytoplasm, nucleus, organelles and the like of cells of tumor tissue. Examples include antigens characteristic of tumor proteins, including proteins encoded by mutated oncogenes; viral proteins associated with tumors; and tumor mucins and glycolipids. Tumors include, but are not limited to, those from the following sites of cancer and types of cancer: lip, nasopharynx, pharynx and oral cavity, esophagus, stomach, colon, rectum, liver, gall bladder, biliary tree, pancreas, larynx, lung and bronchus, melanoma of skin, breast, cervix, uteri, uterus, ovary, bladder, kidney, brain and other parts of the nervous system, thyroid, prostate, testes, Hodgkin's disease, non-Hodgkin's lymphoma, multiple myeloma and leukemia. Viral proteins associated with tumors would be those from the classes of viruses noted above. Antigens characteristic of tumors may be proteins not usually expressed by a tumor precursor cell, or may be a protein which is

normally expressed in a tumor precursor cell, but having a mutation characteristic of a tumor. An antigen characteristic of a tumor may be a mutant variant of the normal protein having an altered activity or subcellular distribution. Mutations of genes giving rise to tumor antigens, in addition to those specified above, may be in the coding region, 5' or 3' noncoding regions, or introns of a gene, and may be the result of point mutations, frameshifts, deletions, additions, duplications, chromosomal rearrangements and the like. One of ordinary skill in the art is familiar with the broad variety of alterations to normal gene structure and expression which gives rise to tumor antigens. Specific examples of tumor antigens include: proteins such as Ig-idiotype of B cell lymphoma, mutant cyclin-dependent kinase 4 of melanoma, Pmel-17 (gp100) of melanoma, MART-1 (Melan-A) of melanoma, p15 protein of melanoma, tyrosinase of melanoma, MAGE 1, 2 and 3 of melanoma, thyroid medullary, small cell lung cancer, colon and/or bronchial squamous cell cancer, BAGE of bladder, melanoma, breast, and squamous cell carcinoma, gp75 of melanoma, oncofetal antigen of melanoma; carbohydrate/lipids such as muc1 mucin of breast, pancreas, and ovarian cancer, GM2 and GD2 gangliosides of melanoma; oncogenes such as mutant p53 of carcinoma, mutant *ras* of colon cancer and HER-2/*neu* proto-oncogene of breast carcinoma; viral products such as human papilloma virus proteins of squamous cell cancers of cervix and esophagus. It is also contemplated that proteinaceous tumor antigens may be presented by HLA molecules as specific peptides derived from the whole protein. Metabolic processing of proteins to yield antigenic peptides is well known in the art; for example see U.S. patent 5,342,774 (Boon et al.).

Preferred tumor antigens of the invention include the Melonoma tumor antigens (e.g., MAGE protein family (MAGE-1, MAGE-2, MAGE-3); MART-1 (peptide 27-35); and gp100); and the Colon carcinoma antigens (e.g., peptides of the mutated APC gene product). Particularly preferred Melanoma tumor antigen sequences are those reported by Slingluff et al., in Curr. Opin. in Immunol. 6:733-

740 (1994):

Gene/protein	MHC	Peptide	SEQ.ID NO.
MAGE-1	A1	EADPTGHSY	1
	Cw1601	SAYGEPRKL	2
MAGE-3	A1	EVDPIGHLY	3
Tyrosinase	A2	MLLAVLYCL	4
		YMNGTMSQV	5
	A24	-------	
gp100/pMel-17	A2	YLEPGPVTA	6
		LLDGTATLRL	7
MART-1/Melan-A	A2	AAGIGILTV	8
		QDLTMKYQIF	20

The MAGE protein family also reportedly has been associated with more than one type of carcinoma: MAGE-1 (Melanoma, thyroid medullary, and small-cell lung carcinoma), MAGE-2 (Melanoma, small-cell lung, colon, bronchial squamous cell, and thyroid medullary carcinoma), and MAGE-3 (Melanoma, small-cell lung, colon, bronchial squamous cell, and thyroid medullary carcinoma). See, also, Morioka, et al., "A Decapeptide (Gln-Asp-Leu-Thr-Met-Lys-Tyr-Gln-Ile-Phe) from Human Melanoma Is Recognized by CTL in Melanoma Patients", J. Immunol. 153:5650 (1994), for additional tumor antigens (e.g., P1A, Connexin 37, MAGE-1, MAGE-3, MART 1/Aa, gp100, Tyrosinase) and/or information relating to the tissue distribution of selected tumor antigens.

Particularly preferred tumor antigens that are peptides of the mutated APC gene product are those reported by Townsend et al., in Nature 371:662 (1994)):

Codon	Mutation	New Sequence	SEQ.ID No.
298	2bp del	SSST/LCTSKADKSSGNQGGNGVFIVVNAWYS	9
540	1bp del	SEDL/TAGYCKCFEEFVLASRCK	10
1068	4bp del	EQRQ/GIKVQLILFILRALMINTSSSNHIL	11
		DSRNVFLHTGHGEPMVQKQIEWVLIMELIKM	
1353	8bp del	HKAV/FRSEISLQKWCSDTQKST	12

1398	1bp del	DSFE/SVRLPAPFRVNHAVEW	13
1420	1bp del	IISP/VIFQIALDKPCHQAEVKHLHHLLK QLKPSEKYLKIKHLLLKRERVDLSKLQ	14
1439	1bp del	RSKT/LHHLLKQLKPSEKYLKIKHLL LKRERVDLSKLQ	15
1446	10bp del	PPQT/GEKYLKIKHLLLKRERVDLSKLQ	16
1488	1bp del	DADT/YYILPRKVLQMDFLVHPA	17
1490	1bp del	DTLL/LLPRKVLQMDFLVHPA	18
1493	11bp del	LHFA/SRWIFLFIQPECSEPR	19

In alternative embodiments, the second targeting moiety is a ligand that selectively binds to a receptor that is expressed on the surface of a cell (preferably a T cell or a B cell). Exemplary receptors which have naturally occurring ligands that can be mimicked by the second targeting moieties of the invention include receptors selected from the following group: CD2, TCR/C3, CD4, CD8, CD10, CD26, CD28, CD40, CD44, CD45, B7.1 and B7.2. According to yet other embodiments, the second targeting moiety is an antibody or antibody fragment that selectively binds to an epitope expressed on the cell surface. The epitope can be a portion of any of the foregoing receptors.

Regardless of the nature of the second targeting moiety target (e.g., protease, receptor, MHC complex, epitope), phage display and other types of combinatorial libraries can be screened in a manner analogous to that described above to identify non-naturally occurring targeting moieties that are useful in forming the compounds of the invention.

There is no requirement that the second targeting moiety be covalently attached to a second reactive group. For example, the second targeting moiety may have sufficient affinity for its binding partner (e.g., an MHC molecule) to permit cross linking between the same or different target molecules on the same or different cells without forming a covalent complex between the second targeting moiety and its targeted binding partner. There also is no requirement that the second reactive group be the same as the first reactive group. Thus, for example,

the compounds of the invention embrace molecules that include a first binding moiety which contains a first reactive group R^1 that is a boronate and a second binding moiety which contains a second reactive group that is a boronate, a phosphonate or a trifluoroalkylketone group.

A linker is covalently coupled to the first and second targeting moieties, P^1 and P^2, in a manner that does not adversely affect the ability of these moieties to bind to their respective targeted binding partners. Exemplary linkers, including a description of linker composition, size, and procedures for coupling the linker to the targeting moieties are described in the detailed description of the invention and in the Examples. In general, such linkers are commercially available and are coupled to the targeting moieties using conventional coupling procedures which are well known to those of ordinary skill in the art.

Certain aspects and uses of the invention are based on the discovery that certain homodimers are capable of stimulating T cells and that this stimulatory ability is, at least in part, dependent upon the length of the linker. In addition, Applicant has discovered that there exists a length between binding moieties (about 20 angstroms) below which this homodimer is no longer capable of stimulating T cells. This ability of any type of homodimer for crosslinking DP IV proteases to stimulate T cells is unexpected in view of published reports that such DP IV homodimers exhibit a T cell inhibitory activity. (See, e.g., PCT/GB94/02615, "DP-IV-Serine Protease Inhibitors", Applicant Ferring V.V. ("Ferring") and the U.S. patent application which claims priority to Ferring). Ferring describes certain symmetric homodimers containing two active-site directed inhibitors of DP-IV linked via the side chains of their amino acid residues. Thus, the invention provides a new use for the homodimer DP-IV inhibitors disclosed in Ferring, namely, the use of the Ferring homodimers for stimulating T cells in a patient in need of such treatment.

Certain concentrations of the homodimers of the invention have been

found to stimulate blood cells. Thus, compounds of the invention are particularly useful for treating HIV+ patients, for example, by contacting the T cells obtained from an HIV+ patient with a therapeutically effective amount of one or more compounds of the invention under conditions that permit blood cell activation. The stimulatory effect on T-cells of these compounds at stimulating concentrations is illustrated in the accompanying Examples. The cells can be contacted with the crosslinking compounds *in vivo* or *ex vivo*. This stimulatory property of the homodimer compounds of the invention is unexpected and could not have been predicted based upon the reported inhibitory effect on the immune system of certain monomers (e.g., the compounds disclosed in Bachovchin '493) and certain homodimers (e.g., PCT/GB94/02615, "DP-IV-Serine Protease Inhibitors", Applicant Ferring V.V. ("Ferring").

The compounds of the invention can be used to inhibit the enzymatic activity of the proteases to which the targeting moieties selectively bind. Thus, the compounds of the invention are useful for inhibiting post-prolyl cleaving enzymes, as well as for inhibiting other serine and cysteine proteases (e.g., chymotrypsin, trypsin, and elastase).

According to another aspect of the invention, methods are provided for modulating immune system function. The compounds of the invention are administered to subjects in need of immune system modulation in amounts effective to modulate immune system function. Modulation of immune system function includes, but is not limited to, increasing immune function such as by stimulating proliferation and specific immune function of blood cells nonspecifically or by specifically stimulating T and/or B cells and/or bone marrow cells, stem cells, early lineage progenitor cells to produce a prophylactic or therapeutic result relating to infectious disease, cancer, and the like. Specifically included is the use of the compounds of the invention, and in particular, homodimers and/or heterodimers for the treatment of disorders characterized by reduced T cell levels *in vivo*, e.g., HIV

and other disorders associated with a compromised immune system. Modulation of immune system function also includes, but is not limited to, decreasing immune function such as by suppressing generally the immune system in transplant recipients or suppressing specifically the immune system to treat autoimmune disease, allergy and the like. In one important embodiment the homodimers and/or heterodimers of the invention are used to stimulate blood cell proliferation, as described in detail below. Specific conditions that may be treated according to the invention are deemed specific independent aspects of the invention and are described in detail in the tables and examples below.

According to yet another aspect of the invention, a method for stimulating T cells is provided. A crosslinking compound of the invention is contacted with the T cells of a subject in need of such treatment in an amount effective to stimulate T cells. The crosslinking compounds that are particularly useful for stimulating T cells are those compounds which crosslink DP IV molecules as described above, including the preferred crosslinking compounds. As noted above, the preferred crosslinking compounds include a linker, L which when positioned between binding moieties results in a minimum length of about 20 angstroms between these moieties. Preferably, the distance between binding moieties is from 20 to 60 angstroms, more preferably from 30 to 50 angstroms.

According to still another aspect of the invention, pharmaceutical preparations are provided. The pharmaceutical preparations contain a crosslinking compound as described above and, optionally, a pharmaceutically-acceptable carrier. Preferably, the pharmaceutical compositions are sterile. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a human or other animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical

compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

According to yet another aspect of the invention, a hetero bivalent compound, having the structure as shown in Fig. 1A, wherein each component of the structure is as defined in Example 2 in reference to this figure, is provided.

An embodiment of this compound is wherein the structures shown in Fig. 1U and Fig. 1V represent, independently, a binding moiety, wherein **R** represents the remainder of the molecule.

By definition, the linker molecule must be capable of linking atom **A1** of the binding moiety on the left of Fig. 1A to atom **A5** of the binding moiety on the right of Fig. 1A.

Other embodiments of this compound include: there are 4 atoms positioned between the group consisting of **D1** and **D2** and **B** of the binding moiety; the binding moiety is in an L-configuration; **Y1, Y2, Y3,** and **Y4** are hydroxyl groups; the **A4** bonded to the **B** is in the L-configuration and the **A5** bonded to the **B** is in the L-configuration; the binding moiety is an L-amino acid residue conjugated to **B**, a boron molecule; the binding moiety is selected from the group consisting of L-Lys-L-boroPro and a derivative of L-Lys-L-boroPro.

Another embodiment of this compound is wherein the linker molecule contains a functional group selected from the group consisting of a carboxylate group, an amino group, a sulfhydryl group, an imidazole group, an alkene group (a carbon atom double bonded to another carbon atom), an acyl halogen group, e.g., an acylchloride, and CH_2X, wherein X represents a halogen (e.g, the two binding moieties are linked when a nucleophilic group displaces the halogen from the functional group of the CH_2X linker molecule); wherein the linker molecule is further defined as having the structure as shown in Fig. 1T and wherein **[G]** contains atoms selected from the group consisting of a carbon, nitrogen, oxygen,

hydrogen and a sulfur atom; [J] is selected from the group consisting of a CH_2 molecule, a chain of carbon atoms, a chain of nitrogen atoms, and a chain of oxygen atoms; and **m**, **p**, and **q** represent an integer from 1 to 50, inclusive; wherein [G] preferably is an **R** group selected from the group consisting of L-amino acid residues selected from the group consisting of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine and D-amino acid residues selected from the group consisting of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine; wherein the linker molecule preferably is selected from the group consisting of hexanedioic acid (adipic acid), EGS, 1,4-diaminobutane, 1,4-dithiobutane, dithiothreitol, lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine; wherein the linker molecule preferably contains at least two amino groups when the binding moieties contain glutamic acid residues; wherein the linker molecule preferably contains at least two amino groups when the binding moieties contain aspartic acid residues; wherein the linker molecule preferably contains at least two sulfhydryl groups when the binding moieties contain cysteine residues; and wherein the linker molecule length ranges from about 30 Å to about 100 Å.

Another aspect of this invention is a compound having the structure as shown in Fig. 1B. In this compound, the binding moieties are identical, e.g., having **A1, A2, A3, and A4** in both binding moieties, and wherein each component of the structure is defined in Example 2 in reference to this Figure.

An embodiment of the compound where only **A1, A2, A3, and A4** appear in both binding moieties, is the structures shown in Fig. 1U and Fig. 1V which represent, independently, a binding moiety, wherein R represents the remainder of the molecule.

Another aspect of this invention is a compound having the structure as shown in fig. 2A, wherein each component of the structure is as defined in Example 4 in reference to this Figure.

An embodiment of this compound includes the structures shown in Figs. 1U and 1V which represent, independently, a binding moiety, wherein R represents the remainder of the molecule.

Other embodiments of this compound include: there are 4 atoms positioned between D and B of the binding moiety; the binding moiety is in an L-configuration; Y1 and Y2 are hydroxyl groups; the A4 bonded to the B is in the L-configuration; the binding moiety is an L-amino acid residue conjugated to B, a boron molecule; and the binding moiety is selected from the group consisting of L-Lys-L-boroPro and a derivative of L-Lys-L-boroPro.

Additional embodiments of this compound is the linker molecule which includes: a functional group selected from the group consisting of a carboxylate group, an amino group, a sulfhydryl group, an imidazole group, an alkene group, an acyl halogen group, and CH_2X, wherein X represents a halogen; the linker molecule which is further defined as having the structure shown in Fig. 1T, wherein [G] contains atoms selected from the group consisting of a carbon, nitrogen, oxygen, hydrogen and a sulfur atom; [J] is selected from the group consisting of a CH_2 molecule, a chain of carbon atoms, a chain of nitrogen atoms, and a chain of oxygen atoms; and m, p, and q represent an integer from 1 to 50, inclusive; wherein [G] preferably is an R group selected from the group consisting of L-amino acid residues selected from the group consisting of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine and D-amino acid residues selected from the group consisting of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine; wherein the linker molecule preferably is selected from the group consisting of adipic acid, between 2 and 15 consecutive amino acid residues, 1,4-diaminobutane, 1,4-dithiobutane, and dithiothreitol; and wherein the linker molecule span ranges from about 30 Å to about 100 Å.

Yet another embodiment of this compound is peptides ranging from

about 7 to 25 amino acids; wherein exemplary peptide include: a) Myelin proteolipid protein peptide; b) Moth cytochrome C peptide; c) tetanus toxin peptide; d) HIV-1 GP 120 peptide; e) myelin basic protein peptide; f) tumor antigenic peptides, and g) antigenic peptides of infectious agents. Preferably, the Myelin proteolipid protein peptide includes human and mouse peptide selected from the group consisting of PLP peptide from the region of 85-159 and in particular, 95-116, and PLP peptide 105-124, PLP peptide 139-151 and PLP peptide 190-209; the Moth cytochrome C peptide is peptide MCC 94-103; the myelin basic protein peptide is MBP peptide 1-11; the tetanus toxin peptide is selected from the group consisting of tetanus toxoid peptide and P2 tetanus toxoid peptide; and the tumor antigenic peptides and antigenic peptides of infectious agents are described elsewhere in the application.

Another embodiment of this compound is where the naturally occurring receptor is a B cell or T cell surface receptor; and the cell surface receptor is selected from the group consisting of TCR/C3, CD2, CD4, CD8, CD10, CD26, CD28, CD44, CD45, CD40, B7.1 and B7.2.

Another aspect of this invention is a compound having the structure as shown in Fig. 1R, wherein each component of the structure is as defined in Example 3 in reference to this Figure.

An embodiment of this compound includes the structures as shown in Fig. 1U and Fig. 1V which represent, independently, a binding moiety, wherein R represents the remainder of the molecule.

Another embodiment of the compound shown in Fig. 1R is wherein (a) $[G]_m$ is the side chain of a D- or L-isomer of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine; (b) **E2** is D- or L- isomer of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine; (c) **E1** and **E3** are selected from the group consisting of an amino moiety and a carboxylic acid moiety; and (d) **E1** and **E3** are distinct from each

other.

Yet another embodiment of the compound shown in Fig. 1R is wherein (a) $[G]_m$ is the side chain of a D- or L- isomer of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine; (b) **E2** is selected from the group consisting of 2-carboxybutyl, 2-carboxypropyl, 2-aminobutyl, 2-aminopropyl, and a hydrocarbon chain with an amino or carboxy side chain; (c) $[J]_p$ and $[I]_q$ represent, independently, hydrocarbon chains; (d) **E1** and **E3** are selected from the group consisting of an amino moiety and a carboxylic acid moiety; and (e) **E1** and **E3** are distinct from each other.

The compounds of the invention, in particular the homodimeric complexes, can be used to stimulate activation or proliferation of human CD26-bearing lymphocytes, by contacting the lymphocytes with a proliferation or activation-inducing concentration of the compound.

The method preferably involves *in vivo* administration of the compound, admixed with a pharmaceutically acceptable carrier such as pharmaceutical, sterile saline. The patient can be any patient who suffers from a disease state characterized by inadequate lymphocyte activation or concentration. Examples of such diseases are HIV infection, kidney failure, cancer (in particular, cancer accompanied by lymphocyte-depleting chemotherapy), and bone marrow disorders which result in depleted lymphocyte populations in the patient. The compound is preferably administered to the patient orally. The compounds can also be used to stimulate proliferation or activation of lymphocytes *in vitro*, e.g., where a patient's autologous lymphocytes are removed, stimulated to increase activation and/or number of lymphocytes, and reinfused into the patient. This method can be used, for example, to increase the number of cytolytic T cells specific for a patient's tumor or T cells in HIV infected patients.

As used herein, crosslinking compound means the compounds described above as well as salts thereof.

These and other aspects of the invention will be described in greater detail below.

All patents, patent applications, references and other documents that are identified in this patent application are incorporated in their entirety herein by reference.

Definitions

By "amino acid" is meant to include imino acid.

By "boroPro" is meant an alpha-amino boronic acid analog of proline bonded to an amino acid to form a dipeptide with boroPro as the C-terminal residue. "BoroPro" is used to designate such an analog having the carboxyl group of proline replaced with a $B(OH)_2$ group, where $(OH)_2$ represents two hydroxyl groups and B represents boron.

By Xaa is meant any amino acid residue, e.g., a lysine residue.

Also, for this invention, "[Lysine-boroProline]$_2$" and "KbP-S-KbP, where S represents a linker spacer" are used interchangeably. "Dimeric KbP with adipic acid as the spacer linker," "[di(L-Lysine-L-boroProline)adipate," and "KbP_2-Adipate" are used interchangeably. Dimeric and bivalent are used interchangeably.

Linker-spacer molecule, cross-linker, cross-linker molecule, linker molecule and linker group are used interchangeably.

By "agonist" is meant a molecule or compound which activates the signaling pathway in question.

By "antagonist" is meant a molecule or compound which inhibits the signaling pathway in question.

"CD26 ligand" is any protein, glycoprotein, lipoprotein or polypeptide that binds to the T cell receptor CD26 and may provide a stimulatory or inhibitory signal.

CD26, Dipeptidyl Peptidase IV (DP IV) and dipeptidyl aminopeptidase IV are used interchangeably. CD26 is a postproline cleaving enzyme with a

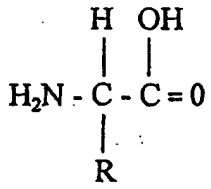
specificity for removing Xaa-Pro (where Xaa represents any amino acid) dipeptides from the amino terminus of polypeptides.

"CD26-specific binding species" means a CD26-specific antibody, fragment or small molecular weight compound that binds to CD26.

"Tethered or coupled α-amino acid" is an α-amino acid with a carbon atom of its side chain tethered, linked or coupled to the N atom of the α-amino group.

By alpha-carbon of an amino acid is the one to which the carboxylic acid group is attached.

By alpha-amino acids is where the amino group is attached to the alpha-carbon. All naturally occurring amino acids are alpha-amino acids or alpha-imino, which means that the amino and carboxylic acid groups are both attached to the same carbon atom. Each amino acid can be thought of as a single carbon atom (the alpha carbon, C) to which there is attached one carboxyl group, one amino group, a side chain denoted R as shown below and a hydrogen.



Wherein: "R" is a side chain; "NH_2" is the alpha amino group; the first carbon (C) attached to the NH_2 group having a hydrogen (H) and an R group attached is the alpha carbon; and the carbon double bonded to an oxygen and a hydroxyl group

(OH) is the alpha carboxyl group.

The NH_2 and COOH groups are used to connect amino acids to one another. The hydroxyl group (OH) of one amino acid on the carboxyl end and the hydrogen (H) on the N terminus are removed (H_20) when two amino acids are linked together. To form a protein, the amino group of one amino acid reacts with the carboxyl group of another by the elimination of water; the resulting chemical bond is called a peptide bond.

By "peptides" is meant a small molecule, e.g., usually containing less than 50 amino acid residues, which do not generally possess a well-defined three-dimensional structure.

By Å or "Anstrom" is meant 10^{-10} M. The relative bond size used in different peptides is as follows: N-H: 1.0 Å; C-H: 1.1 Å; C double bonded to 0: 1.2 Å; N-CO: 1.3 Å; C-O: 1.4 Å; C-C: 1.5 Å; Alanine: 6 Å; Benzene: 6 Å; water: 4 Å; and Phenylalanine: 7 Å.

Pharmaceutical preparations and modes of administration are described herein.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figs. 1A-1V are diagrams showing the general structures of several preferred homobivalent and homomultivalent compounds or are diagrams of components needed for these structures:

Fig. 1A is a diagram of a general bivalent template with non-identical binding moieties.

Fig. 1B is a diagram of a general homobivalent template.

Fig. 1C is a diagram of a general bivalent template with amino linkages using a dicarbonyl linker.

Fig. 1D is a diagram of a bivalent example with amino linkages using an adipoyl linker.

Fig. 1E is a diagram of a homobivalent example with amino linkages using an adipoyl linker [(Lysine-boroPro)$_2$Adipate].

Fig. 1F is a diagram of another homobivalent example with amino linkages using an adipoyl linker.

Fig. 1G is a diagram of a general bivalent template with a carboxyl linkage using a diamino linker.

Fig. 1H is a diagram of a bivalent example with carboxyl linkages using a 1,4-Diaminobutane linker.

Fig. 1I is a diagram of a bivalent example with carboxyl linkages using a 1,4-Diaminobutane linker [(Aspartyl-boroProline)$_2$1,4-Diaminobutane].

Fig. 1J is a diagram of another bivalent example with carboxyl linkages using a 1,4-Diaminobutane linker.

Fig. 1K is a diagram of a general bivalent template with disulfide linkages using a dithiol linker.

Fig. 1L is a diagram of a bivalent example with disulfide linkages using a 1,4-Dithiobutane linker.

Fig. 1M is a diagram of another bivalent example with disulfide linkages using a 1,4-Dithiobutane linker.

Fig. 1N is a diagram of another bivalent example with disulfide linkages using a dithiothreitol linker [(Cysteine-boroProline)$_2$dithiotheitol].

Fig. 1O is a diagram of a general bivalent template with imidazole linkages using a dicarbonyl linker.

Fig. 1P is a diagram of another bivalent example with imidazole linkages using a dicarbonyl linker.

Fig. 1Q is a diagram of a bivalent example with imidazole linkages using an adipoyl linker: (Histidine-boroProline)$_2$Adipate.

Fig. 1R is a diagram of a general homofunctional polymeric crosslinker template.

Fig. 1S is a diagram of a homotrimeric example using adipoyl linkers.

Fig. 1T is a diagram of a linker molecule template.

Fig. 1U is a diagram of a binding moiety containing A1, A2, A3, and A4.

Fig. 1V is a diagram of a binding moiety containing A5, A6, A7, and A8.

Figs. 2A-2C are diagrams showing the general formula of several preferred heterobivalent compounds:

Fig. 2A is a diagram of a general heterobivalent template.

Fig. 2B is a diagram of a heterobivalent example coupling a binding moiety to an MCC peptide (94-103).

Fig. 2C is a diagram of a heterobivalent example coupling a binding moiety to a PLP peptide (139-151).

Fig. 3 is a diagram showing the synthesis of adipoyl $(Lys\text{-}boroPro)_2$, a homobivalent derivative of Lys-boroPro.

Fig. 4 is a graph showing a dose response curve observed with lower concentrations of KbP_2-Adipate on anti-CD3 mAb stimulation of H9 cells.

Fig. 5 is a graph showing a dose response curve observed with higher concentrations of KbP_2-Adipate on anti-CD3 mAb stimulation of H9 cells. Drug concentration is read as 10^XM.

Fig. 6 is a graph showing a dose response curve for anti-1F7.

Fig. 7 is a diagram showing intermolecular reactions that may occur at higher concentrations of KbP_2-Adipate.

Fig. 8 is a diagram showing Lys-boroPro linked to Myelin Proteolipid Protein (PLP) Peptide 139-151.

Fig. 9 is a graph comparing the effects heterobivalent coupled KbP-S-

MCC and uncoupled MCC 94-103 have on IL-2 production in 2B4 cells.

Fig. 10 is a diagram showing the structures of the open and cyclized forms of Xaa-boroProline inhibitors.

Figs. 11A-11D are diagrams of different examples of bivalent compounds containing an olefin group. Fig. 11D is a fluoroolefin isostere of Xaa-boroProline.

Fig. 12 is a graph illustrating the effect of $(KbP)_2$-EGS at varying concentrations on IL-2 production by H9 cells.

Fig. 13 is the structure of EGS, the spacer-linker molecule used in the homodimer $(KbP)_2$-EGS used in the experiments of Fig. 12.

Detailed Description

Synthetic, low molecular weight, bivalent and multivalent crosslinking compounds with agonistic activity are designed and developed. To have agonist activity, the molecule needs to be able to induce the association of receptors of a specific class in a manner similar to that induced by its natural ligand. These agonistic molecules therefore need to be at least bidentate. Additionally, the individual binding units must be properly spaced for the desired association to occur.

The homobivalent (homodimeric), homomultivalent and heterobivalent (heterodimeric) compounds of this invention represent a new class of biological modulators which can be used as therapeutic or diagnostic agents or both.

T Cell Surface Receptors

T cell surface receptors and their naturally occurring ligands are used herein as examples to demonstrate how different homobivalent, homomultivalent, heteromultivalent, and heterobivalent synthetic crosslinking compounds function; these examples are therefore not intended to limit the invention. The molecule,

Xaa-boroPro, which has been shown to have high affinity for the CD26 T cell surface receptor (See above discussion), can be a component molecule of the homobivalent, heteromultivalent, homomultivalent, or heterobivalent compounds outlined herein.

Biochemistry of CD26 T Cell Surface Receptor

CD26 is a highly glycosylated type two transmembrane protein. It exists as a dimer with a subunit molecular weight of about 110 kDa. The cDNAs encoding the human, mouse and rat proteins have been cloned and sequenced (D. Darmoul, et al., "Dipeptidyl peptidase IV (CD26) gene expression in enterocyte-like colon cancer cell lines HT-29 and Caco-2: Cloning of the complete human coding sequence and changes of dipeptidyl peptidase IV mRNA levels during cell differentiation," J.Biol.Chem. 267, 4824-4833 (1992); D. Marquet, et al., "cDNA cloning for mouse thymocyte-activating molecule: A multifunctional ecto-dipeptidyl peptidase IV (CD26) included in a subgroup of serine protease." J.Biol.Chem. 267, 2200-2208 (1992); T. Tanaka, et al., "Cloning and functional expression of the T cell activation antigen CD26," J.Immunol. 149, 481-486 (1992); published erratum appears in J.Immunol. 50(5):2090 (Mar 1993)). Human CD26 cDNA encodes a 766 amino acid polypeptide (having a molecular weight of 88,300) and the mouse cDNA encodes a 760 amino acid polypeptide (87,500 molecular weight). The sequences of the mouse, rat and human CD26 share up to 98% homology. Most of the CD26 molecule resides outside of the cell by being anchored to the cell plasma membrane through a 22 amino acid hydrophobic domain on the N terminus and only a small, six amino acid N terminal tail projects into the cytoplasm.

Among lymphoid cells, CD26 is found mainly on the surface of CD4+ T cells where it is believed to have important roles in T cell activation pathways (see section on CD26 and T cell function below). CD26 is also found on a small

fraction of CD8+ cells. CD26 has been shown to be identical with an enzyme known as dipeptidyl peptidase amino peptidase Type IV (DP IV, sometimes also abbreviated DPP IV or DAP IV).

Enzymology of CD26: Active Site Structure and Inhibitor Design

The catalytic activity thus far identified for CD26 associated DP IV protease activity involves the cleaving of a dipeptide unit from the free amino terminus of polypeptides and proteins. DP IV shows a strong preference for cleaving after a proline residue, i.e., a proline in the penultimate position from the amino terminus. A free amino terminus appears to be a requirement but the enzyme displays little preference for any particular amino acid in this position (J. Heins, et al., "Mechanism of proline-specific proteinases: (I) Substrate specificity of dipeptidyl peptidase IV from pig kidney and proline-specific endopeptidase from Flavobacterium meningosepticum," Biochimica Et Biophysica Acta 954, 161-169 (1988)). DP IV can be regarded as a postproline cleaving aminopeptidase with a specificity for removing N-terminal Xaa-Pro dipeptides where Xaa can be any amino acid. However, DP IV will also remove amino terminal Xaa-Ala dipeptides, although much less effectively. The enzyme does, however, require that some amino acid be N terminal to proline because boroPro itself is not an effective inhibitor. The P2 residue is probably required only for presenting a free amino group in the appropriate geometrical arrangement with respect to the proline bond to be cleaved because the addition of a group to the P2 N terminus, such as Ac, CBZ, or Fmoc, abolishes the inhibitory potency (G.R. Flentke, et al., "Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhhibitors to examine the role of DP-IV in T-cell function," PNAS (USA) 88, 1556-1559 (1991)). This indicates that the active site is constructed to recognize a proline residue at P1 and a free amino group at P2. The P2 amino acid side chain is probably directed away from the enzyme and free in solution. P1 refers to the residue on the N-terminal side of the sessile bond. P2 refers to the residue on the

N-terminal side of P1.

CD26 and T Cell Function

The work of Schon et al. was among the first to implicate DP IV as important to T cell function (E. Schon, et al., "Dipeptidyl peptidase IV in the immune system. Effects of specific enzyme inhibitors on activity of dipeptidyl peptidase IV and proliferation of human lymphocytes," Biological Chemistry Hoppe Seyler 372, 305-311 (1991); E. Schon, et al., "The dipeptidyl peptidase IV, a membrane enzyme involved in the proliferation of T lymphocytes," Biomedica Biochimica Acta 44, (1985); E. Schon, et al., "Dipeptidyl peptidase IV in human T lymphocytes. An approach to the role of a membrane peptidase in the immune system," Biomedica Biochimica Acta 45, 1523-1528 (1986); E. Schon, et al., "The role of dipeptidyl peptidase IV in human T lymphocyte activation. Inhibitors and antibodies against dipeptidyl peptidase IV suppress lymphocyte proliferation and immunoglobulin synthesis in vitro," European Journal of Immunology 17, 1821-1826 (1987); E. Schon et al., "Dipeptidyl peptidase IV in human T lymphocytes. Impaired induction of interleukin 2 and gamma interferon due to specific inhibition of dipeptidyl peptidase IV," Scandinavian Journal of Immunology 29, 127-132 (1989)). This work reported that DP IV inhibitors and anti-DP IV polyclonal antibodies suppressed T cell activation in culture. It has been demonstrated that Pro-boroPro and Ala-boroPro inhibited an immune response *in vivo* in mice such that antibody production in response to an antigen challenge was reduced; this was direct evidence for a role for DP IV/CD26 in immune function *in vivo*.

Also, most of the evidence implicating CD26/DP IV as important to proper T cell function and immune system regulation comes from studies of the effects of various anti-CD26 mAbs on T cell functions. Because Abs are naturally bivalent, they are often able to mimic the natural ligand in inducing association of the receptor, e.g., an agonistic effect, which may be a dimerization or an

aggregation. If an antibody fails to correctly mimic the natural-ligand induced association, it will then often block the interaction with the natural ligand and therefore show an inhibitory or antagonistic activity.

The anti-CD26 mAbs thus far identified have either activating, inhibitory, or both effects on various T cell responses. These results show that CD26 is an association-activated co-stimulatory T cell receptor.

The role for CD26 as an association-activated, co-stimulatory molecule has been recently confirmed in experiments in which the gene for CD26 was transfected into Jurkat T cell lines (T. Tanaka, et al., "Cloning and functional expression of the T cell activation antigen CD26," published erratum appears in J. Immunol., 150(5):2090 (Mar 1993); J.Immunol. 149, 481-486 (1992)).

The results indicated that mAb-mediated cross linking of CD26 on CD26+ Jurkat cell resulted in enhanced Ca^{2+} mobilization and IL-2 production in response to anti-CD3 suboptimal stimulation in the presence of phorbol esters. Untransfected Jurkat cells do not express CD26 and do not produce IL-2 in significant amounts in response to anti-CD26 costimulation with suboptimal anti-CD3 treatment in the presence of phorbol esters.

Mechanisms of CD26 Mediated Signal Transduction

CD26 has only a short six amino acid cytoplasmic tail. This argues against signal transduction via the cytosolic domain as is often the case for other cell surface receptors. CD26 may participate in T cell signal transduction through two hypothetical mechanisms: (1) through its association with other molecules in the membrane, and (2) through its DP IV protease activity.

EXAMPLES

Introduction

Throughout this application and in particular, in each of the Examples and drawings, particular embodiments are described and illustrated. It is to be

understood that any of the reactive groups disclosed herein can be substituted for the particular reactive groups (e.g., boronyl group) shown in the drawings or described in the particular Examples.

EXAMPLE 1
GENERAL SYNTHESIS OF HOMOBIVALENT, HOMOMULTIVALENT AND MULTIVALENT CROSSLINKING COMPOUNDS

The synthesis of bivalent or multivalent compounds or agents outlined herein involves essentially very similar chemistry. These bivalent and multivalent compounds are designed such that they induce associations between naturally occurring receptors, e.g., an association between a T cell surface CD26 receptor with itself (homobivalent) or an association between three T cell surface (CD26 receptors (homomultivalent, e.g., homotrivalent) or an association between CD26 receptor with the T cell receptor (TCR/CD3) or with the CD4 receptor (heterobivalent).

For the most part, straightforward peptide coupling chemistry is employed. The standard peptide coupling chemistry methods and procedures used in this invention are readily available. Examples of books using these methods include, but are not limited to, the following citations incorporated herein by reference: P.D. Bailey, An Introduction to Peptide Chemistry, Ed.: John Wiley & Sons, 1990; Miklos Bodansky, Peptide Chemistry, A Practical Textbook, Ed.: Springer-Verlag, 1988; Miklos Bodansky, Principles of Peptide Synthesis, "Reactivity and Structure Concepts in Organic Chemistry," Volume 16, Ed.: Springer-Verlag, 1984; and Miklos Bodansky, Principles of Peptide Synthesis, "Reactivity and Structure Concepts in Organic Chemistry," Volume 21, Ed.: Springer-Verlag, 1984.

1. Homomultivalent Compounds: General Structure

The homobivalent and homomultivalent compounds taught herein can either start with the general diagram for a bivalent template with non-identical

binding moieties as shown in Fig. 1A or with the general diagram for a homobivalent template as shown in Fig. 1B. Thus, both sides of the chemical structure as shown in Figs. 1A and 1B are binding moieties. Figs. 1C-1T are diagrams showing the structure of several preferred bivalent and multivalent compounds. Descriptions of the figures are provided above in the Brief Description of the Drawings.

2. Heterobivalent Compounds: General Structure

The heterobivalent and heteromultivalent compounds and agents taught herein may begin with the following general diagram as shown in Fig. 2A, the general formula for a heterobivalent compound. Figs. 2A-2C are diagrams showing the general formula of several preferred heterobivalent compounds: Fig. 2A is a diagram of a general heterobivalent template; Fig. 2B is a diagram of a heterobivalent example coupling a binding moiety to an MCC peptide (94-103) using a compatible linker, e.g., an AAAAAA (SEQ ID NO: 21) linker group where A is L-alanine or D-alanine; and Fig. 2C is a diagram of a heterobivalent example coupling a binding moiety to a PLP peptide (139-151) using a compatible linker, e.g., an AAAAAA linker group where A is L-alanine or D-alanine.

For this invention, peptide, polypeptide, or fragment thereof, are used interchangeably and is defined to include a chain of amino acids ranging from about 3 to 50 residues, preferably from about 3 to 25 residues in length. More preferably, optimal size is on the order of about 10-18 amino acid residues in length.

Known antigenic peptides for any of the following autoimmune diseases, infectious diseases, allergic diseases, or cancers listed below and elsewhere in this document (without limitation) could be coupled to a bivalent template (see Fig. 2A), e.g., Xaa-boroPro, to form a heterobivalent compound (heterodimeric) molecule that could be used in this invention to treat that disease. Thus, once coupled or linked to the bivalent or multivalent template (see Fig. 2A), these peptides form different heterobivalent compounds which can alter biological

activity (increased or reduced) as a result of bivalent interaction inducing the association of two receptors.

AUTOIMMUNE DISEASES AND KNOWN ANTIGENIC PEPTIDES

AUTOIMMUNE DISEASE	AUTOANTIGEN	REFERENCE
Addison's disease	adrenal specific antigen with a molecular weight of 18-24 kDa	Freeman, et al., Clinical & Experimental Immunology, 88(2):275-279 (May 1992)
hemolytic anemia	internal membrane protein and the integral red blood cell protein Band-3	Perry, et al., European Journal of Immunology, 26(1):136-141 (January 1996)
antiphospholipid syndrome	epitopes on the fifth domain of beta 2-glycoprotein I	Wang, et al., Journal of Immunology, 155(3):1629-1636 (August 1, 1995)
rheumatoid arthritis	deglycosylated aggrecan peptide spanning the chondroitin sulphate domain	Goodstone, et al., Annals of the Rheumatic Diseases. 55(1):40-46 (January 1996)
herpetiformis dermatitis	strong association with specific human histocompatibility leukocyte antigens DR3, Dqw2, and DPw1	Hall et al., Seminars in Dermatology, 10(3):240-245 (September 1991)
diabetes mellitus, insulin dependent	"The mapping of most of the genetic risk (or disease resistance) to specific alleles in the major histocompatibility locus (MHC class II) has direct functional implications for our understanding of autoimmunity in diabetes and directly implies that presentation of a likely narrow set of peptides is critical to the development of autoimmunity."	Karges, et al., Molecular Aspects of Medicine, 16(2):79-213 (1995)

allergic encephalomyelitis (major animal model for human multiple sclerosis)	proteolipid protein residues 139-151	Kuchroo, et al., Journal of Immunology, 148(12):3776-3782 (June 15, 1992)
glomerulonephritis, IgA	antibodies directed against endothelial cells	Wang et al., Nephrology, Dialysis, Transplantation, 7(8):805-810 (1992)
glomerulonephritis, membranous	"Towards defining antigens in human membranous nephropathy"	Brenchley, et al., Nephrology Dialysis, Transplantation, 7 Suppl. 1:21-24 (1992)
Goodpasture's syndrome	alpha 3 chain of type IV collagen	Kalluri, et al., Journal of the American Society of Nephrology, 6(4):1178-1185 (October 1995)
Grave's disease	thyroid stimulating hormone receptor	Mullins, et al., Journal of Clinical Investigation, 96(1):30-37 (July 1996)
Lambert-Eaton myasthenic syndrome	N- and L-type calcium channels	el Far, et al., Journal of Neurochemistry, 64(4):1696-1702 (April 1995)
lupus erythematosus, systematic	small nuclear ribonucleoprotein C, residues 117-126	James, et al., Clinical & Experimental Rheumatology, 13(3):299-305 (May-June 1995)
multiple sclerosis	the small heat-shock protein alpha B-crystallin	van Noort, et al., Nature, 375(6534):798-801 (June 29, 1995)
myasthenia gravis	muscle nicotinic acetylcholine receptor	Protti, et al., Immunology Today, 14(7):363-368 (July 1993)
neuritis, experimental allergic	the homophilic cell adhesion molecule p0 glycoprotein, residues 56-71 and 180-199	Linington, et al., European Journal of Immunology, 22(7):1813-1817 (July 1992)
sympathetic opthalmia	autoantigen unknown but immunosuppressive therapy effective	Chan, et al., Archives of Ophthalmology, 113(5):597-600 (May 1995)

pemphigoid, bullos	an epidermal hemidesmosomal glycoprotein named BP180 (human) and mBP180 (mouse). In the mBP180 ectodomain, an antigenic site comprised of 9-12 residues designated mBP1 is recognized by pathogenic sera.	Liu, et al., Journal of Immunology, 155(11):5449-5454 (December 1995)
pemphigus	desmoglein 3 (DG) residues 190-204	Wucherpfennig, et al., Proceedings of the National Academy of Sciences of the United States of America, 92(25):11935-11939 (December 1995)
polyendocrinopathies, autoimmune	steroidogenic enzymes P450scc, P450c17, and P450c21	Uibo, et al., Journal of Autoimmunity, 7(3):399-411 (June 1994)
purpura, thrombocytopenic, idiopathic	platelet surface glycoproteins Ib, IIB, IIIa with molecular weights 160, 135 88 kDa respectively.	Kokawa, et al., European Journal of Hematology, 50(2):74-90 (1993)
Reiter's disease	platelet unknown but antibiotic treatment is effective	Huges, et al., Seminars in Arthritis & Rheumatism, 24(3):190-210 (December 1994).
stiff-man syndrome	linear NH2-terminal epitope of glutamic acid decarboxylase 65	Daw, et al., Journal of Immunology, 156(2):818-825 (January 15, 1996)
thyroiditis, autoimmune	thyroid peroxidase	Chazenbalk, et al., Journal of Clinical Investigation, 92(1):62-74 (July 1993)

INFECTIOUS DISEASES AND KNOWN ANTIGENIC PEPTIDES

INFECTIOUS DISEASE	ANTIGENIC PEPTIDE(S)	REFERENCE
HIV-1 and HTLV-1	Synthetic Peptide Immunogens	Hart, et al., Pharmaceutical Biotechnology, 6:821-845 (1995)
Malaria	SPF(66)n	Lopez, et al., Vaccine, 12(7):585-591 (1994)
Schistosomisis	Triose-Phosphate Isomerase	Reynolds, et al., Journal of Immunology 152(1):193-200 (January 1, 1994)
HIV-1	Chemically Defined Synthetic Vaccine	Nardelli, et al., Journal of Immunology, 148(3):914-920 (February 1, 1992)
Toxoplasmosis	Toxoplasma Gondii P30 Antigen	Darcy, et al., Journal of Immunology, 149(11):3636-3641 (December 1, 1992)
Malaria	Epitopes of Cs and RESA Proteins	Ritu, et al., Vaccine, 10(11):761-765 (1992)

ALLERGIC DISEASES AND KNOWN ANTIGENS

ALLERGIC DISEASE	ANTIGEN(S)	REFERENCE
Cedar Allergy	(Cry j 1) - derived peptide	Ikagawa, et al., Journal of Allergy & Clinical Immunology, 91(1 Pt 1):53-64 (January 1996)
Penicillin Allergy	beta-lactam ring	Brander, et al., Journal of Immunology, 155(5):2670-2678 (September 1. 1995)
House Dust Mite Allergy	Der p 2 allergen	O'Brien, et al., Immunology, 86(2):176-182 (October 1995
Ragweed Allergy	Amb a 5 and Amb t 5 allergens	Greenstein. et al.. Journal of Immunology 155(10):5064-5073 (November 15. 1995)
Soybean Allergy	Alpha-subunit of beta-conglycinin	Ogawa, et al., Bioscience, Biotechnology & Biochemistry, 59(5):831-833 (May 1995)
Bee Venom Allergy	Phospholipase A2	Dudler. et al., European Journal of Immunology. 25(2):538-542 (February 1995)
Rye Grass Allergy	T cell epitopes of the major fraction	Bungy, et al.. European Journal of Immunology. 24(9):2098-2103 (September 1994)
Egg Allergy	Epitope of Ovalbumin	Shimojo, et al., International Archives of Allergy & Immunology. 105(2):155-161 (October 1994)
Dermatophagoides Pteronyssinus Allergy	Peptides of Der p 11	Okana, et al., Allergy. 49(6):436-441 (July 1994)

Listed below are examples of different known peptides with known specificity and high affinity for different T cell surface receptors. These peptides (P) can be coupled to Xaa-boroPro or coupled to another binding moiety as shown in Fig. 2A to form different heterobivalent compounds exhibiting altered biological activity (increased or reduced as a result of the specific binding to the specific receptor.

HETEROVIBALENT PEPTIDES

PEPTIDE	SPECIFIC FOR
Myelin Proteolipid Protein (PLP 139-151)	TCR*/CD3
Moth Cytochrome C peptide (MCC)	CD3
Colony Stimulating Factor Peptide	TCR/CD3
Stem Cell Factor Peptide	TCR/CD3
HIV-1 GP 120 Peptide	CD4
HIV-1 GP 120	CD4
P2 Peptide of Tetanus Toxoid	TCR/CD3
Multiple Sclerosis Peptide	CD4
Peptide Analog of Myelin Basic Protein	TCR
Myelin Proteolipid Protein	TCR
HIV-1 gp120	TCR
Tetanus Toxoid	TCR
Stem Cell Factor (SCF)	SCF Receptor
Cytochrome c	TCR
Tetradecapeptide Epitope of Myelin Basic Protein	TCR
Colony-Stimulating Factor (CSF)	CSF-Receptor

TCR: T cell Surface Receptor

3. Synthesis of H-boroPro and Xaa/Lys-boroPro

Homobivalent, homomultivalent, heterobivalent, and heteromultivalent compounds can begin with the synthesis of H-boroPro and LysboroPro as taught herein. Use of H-boroPro and Lys-boroPro are for example purposes only, and is not intended to limit the scope of this invention. Thus, Xaa/Lys-boroPro is an example of a molecule that can be used to form a binding moiety of a bivalent, homobivalent, homomultivalent, or heterobivalent compound as taught herein.

Standard peptide coupling chemistry methods and procedures used in this invention are taught in the books identified at the beginning of this Example. In particular, H-boroPro was prepared by the synthetic route previously developed and described (G.R. Flentke, et al., "Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhibitors to examine the role of DP-IV in T-cell function," PNAS (U.S.A.) 88, 1556-1559 (1991); also described in United States Patent No. 5,462,928). Alternatively, H-boroPro may be produced by a new procedure (Kelly, T.A., et al., "The efficient synthesis and simple resolution of a proline boronate ester suitable for enzyme inhibition studies," Tetrahedron 49, 1009-1016 (1993)). Both of these synthetic routes yield racemic H-boroPro pinanediol.

Stereochemically pure L, L and L, D diastereomers of Z-Lys-boroPro were prepared by first resolving racemic H-boroPro through crystallization with optically active blocking protecting groups ((1S, 2S, 3R, 5S)-+-pinanediol isomer) followed by coupling the isotopically pure L-boroPro and D-boroPro to the stereochemically pure L isomer of lysine (See United States Patent No. 5,462,928). Alternatively, the L,L and L,D diastereomers of Lys-boroPro were prepared in high optical purity by coupling racemic H-boroPro by L-Lys and separating the resulting diastereomeric Z-Lys-boroPro-diester into its component L,D and L,L diastereomers using reverse phase HPLC as previously described for diastereomeric Pro-boroPro (W.G. Gutheil and W.W. Bachovchin, "Separation of L-Pro-DL-boroPro into Its Component Diastereomers and Kinetic Analysis of Their Inhibition of Dipeptidyl Peptidase IV. A New Method for the Analysis of Slow, Tight-Binding Inhibition," Biochemistry 32, 8723-8731 (1993)). Thus, there are several

routes through which to obtain any of the four possible stereoisomers of Lys-boroPro. However, only the DP IV inhibitory L,L isomer and the DP IV non inhibitory L,D isomer were normally prepared for use as a control in the immunological experiments. In general, the derivatives prepared herein use optically pure diastereomers and therefore contain only either the L,L or the L,D isomer of Lys-boroPro.

Once prepared, these Xaa-boroPro compounds are coupled to other Xaa-boroPro compounds, e.g., itself, to form a homobivalent (homodimeric) or multivalent compound or coupled to a non-Xaa-boroPro peptide thereby forming a heterobivalent (heterodimeric) or multivalent compound.

EXAMPLE 2
SYNTHESIS OF HOMOBIVALENT AND HETEROBIVALENT COMPOUNDS AND ASSESSMENT OF FUNCTIONAL ACTIVITY

Homobivalent, low molecular weight compounds capable of inducing an association between two CD26 receptors are taught in this Example. Homobivalent synthetic crosslinking compounds have the properties normally associated with an antibody, i.e., high affinity, and specificity for CD26, and an ability to induce crosslinking. Thus, any experiment with an anti-CD26 monoclonal antibody can be performed with one or more of the homobivalent compounds, e.g., immunoprecipitations.

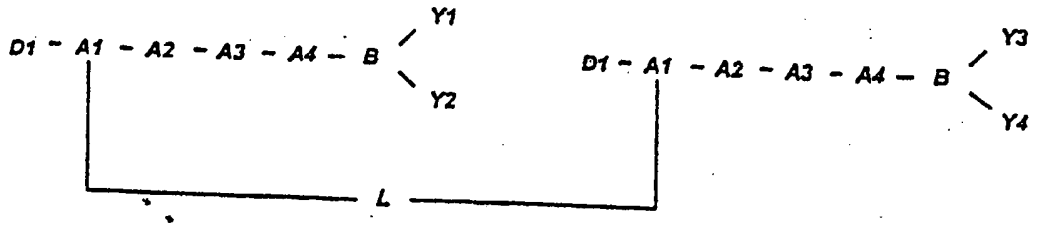
The synthetic ligands, however, have some properties that make them complimentary to anti-CD26 mAbs. These include: (i) their binding epitope is the DP IV active site; (ii) they exhibit cross-species specificity; and (iii) they offer flexibility in adjusting the spacing between the binding sites and the construction of chimeric or heterobidentate structure.

I. Synthesis of Homobivalent Xaa-boroPro Derivatives and Heterobivalent Compounds

To produce molecules which induce the association between one cell surface CD26 with another cell surface CD26 and also retain DP IV inhibitory activity, a series of different homobivalent derivatives of Lys-boroPro will be linked via their ϵ-amino groups by a linker-spacer molecule containing two carboxylic acid groups, e.g., a six carbon

linker spacer or linker group, using conventional peptide coupling methods (see Fig. 3). Fig. 3 is a diagram showing the synthesis of adipoyl (Lys-boroPro)$_2$, a homobivalent derivative of Lys-boroPro. This linkage method included coupling benzyloxycarbonyl-lysine-boroPro-diester (Z-lys-boroPro-diester) to a linker molecule, e.g., adipic acid or hexanedioic acid (HOOC(CH$_2$)$_4$COOH). The diester protecting group on the boronyl moiety can either be pinacol or pinanediol.

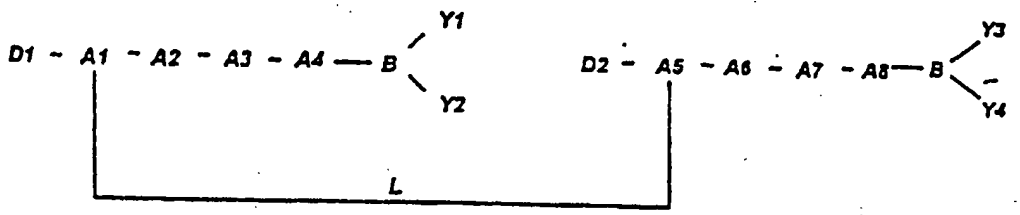
The general structure for this homobivalent (homodimeric) compound is shown in Fig. 1B:



wherein D1, is independently selected from the group consisting of NH and NH$_2$, wherein N represents any isotope of nitrogen, wherein H represents an isotope of hydrogen; "~", independently, is selected from the group consisting of a single bond and a double bond; B represents, independently, any isotope of boron; A1 is, independently, selected from a group consisting of a C, a CS moiety and an N, wherein C represents any isotope of carbon, wherein X represents any atom capable of forming a single bond with C; each A2, A3, and A4 are, independently, selected from a group consisting of a CX moiety, a CXZ moiety, a CZ moiety, an NX moiety, and an O, wherein X and Z, are, independently, selected from the group consisting of any atom capable of forming a single bond and any atom capable of forming a double bond with C or N and wherein O represents any isotope of oxygen; wherein each Y1, Y2, Y3, and Y4 are, independently, selected from the group consisting of a hydroxyl moiety and any reactive moiety that converts to a hydroxyl moiety under physiologic conditions; and L represents a linker molecule (i) having a

molecular weight ranging between about 100 daltons and about 2000 daltons, (ii) having a span ranging from about 20 Å to about 300 Å, and (iii) containing a chain of atoms selected from the group consisting of a combination of C, O, N, S, and Ph atoms, connected by single bonds or by double bonds in a manner that does not violate the laws of chemistry and wherein S represents any isotope of sulfur and Ph represents any isotope of phosphorous.

The structure as shown above need not be identical, in that it can have the general structure as shown in Fig. 1A:



wherein D1 and D2, independently, are selected from the group consisting of NH and NH_2, wherein N represents any isotope of nitrogen, wherein H represents any isotope of hydrogen; "~", independently, is selected from the group consisting of a single bond and a double bond; B represents, independently, any isotope of boron; A1 and A5 are, independently, selected from a group consisting of a C, a CX moiety and an N, wherein C represents any isotope of carbon, wherein X represents any atom capable of forming a single bond with C; each A2, A3, A4, A6, A7, and A8 are, independently, selected from a group consisting of a CX moiety, a CXZ moiety, a CZ moiety, an NX moiety, and an O wherein X and Z, are, independently, selected from the group consisting of any atom capable of forming a single bond and any atom capable of forming a double bond with C or N and wherein O represents any isotope of oxygen; wherein each Y1, Y2, Y3, and Y4 are, independently, selected from the group consisting of a hydroxyl moiety and any reactive moiety that converts to a hydroxyl moiety under physiologic conditions; and L represents a linker molecule (i) having a molecular weight ranging between about 100 daltons and about 200 daltons, (ii) having a span ranging from about 20 Å to about 300 Å, and (iii) containing a chain of atoms selected from the group consisting of a combination of C, O, N, S, and Ph atoms, connected by single bonds or by double bonds in a manner

that does not violate the laws of chemistry and wherein S represents any isotope of sulfur and Ph represents any isotope of phosphorous.

The coupling reaction can be achieved by any of several standard peptide coupling methodologies. For example, the Lys-boroPro homobivalent (homodimeric) derivative was prepared by reacting protected Lys-boroPro in anhydrous THF with the acid chloride form of adipic acid, i.e., adipoyl chloride, which is commercially available (Aldrich Co.). Following coupling, the N terminal Z protecting group was removed by catalytic hydrogenation. Deprotection of the boronyl group was achieved by transesterification with phenyl boronic acid and extracted using a two phase, low pH water solution/organic solvent.

II. Determination of Optimal Chemical Spacer Linkers

To determine the optimal spacer linker segment or linker molecule for inducing the association of one cell surface CD26 with another cell surface CD26 receptor, a series of bivalent, dimeric Lys-boroPro derivatives with varying length spacer segments will be prepared (See discussion below on linker molecules). A wide assortment of dicarboxylic spacer linker molecules are commercially available. This includes linker molecules which have various internal heteroatoms and other functional groups, in addition to the terminal carboxylic groups, e.g., ethylene glycolbissuccinate ("EGS", shown in Fig. 13).

For example, using EGS in place of adipic acid provides a bivalent compound with a spacer of about twice the length of the adipoyl moiety. Also, the internal heteroatoms confer improved water solubility over a straight chain hydrocarbon of similar length.

Fig. 13 gives the structure of EGS, which was used as the linker molecule joining two KbP monomers, to form $(KbP)_2$ EGS. The synthesis of this homodimer was carried out in a manner analogous to that described herein for KbP_2 adipate, using appropriate modifications. EGS is commercially available from a variety of chemical supply companies.

Both $(KbP)_2$ adipate and $(KbP)_2$ EGS were used in experiments with the T cell line H9 to determine their effects on activation (as measured by Il-2 production) and/or

proliferation. Some of these experiments employed the homodimeric molecules as co-stimulatory factors with other, known T cell stimulatory factors such as the monoclonal antibody OKT3. The results of these experiments are shown in Figs. 5 and 12.

III. Experimental Methods

To determine the effect KbP_2-Adipate had on anti-CD3 mAb stimulation of H9 cells, e.g., stimulation of suppressing IL-2 production, the following protocol was used.

In quadruplicate wells, H9 cells were preincubated with KbP_2-Adipate for 0, 30, 60, or 150 min. After the preincubation, the cells were seeded into a 96 well flat bottom plate pre-coated with anti-CD3 monoclonal antibody OKT3 at 1,000, 5,000, 10,000, 20,000, 50,000, or 100,000 times dilution of stock. After 24 hr, the cells were lysed by freezing at 4°C. IL-2 concentration in the H9 cells lysed was bioassayed using the IL-2 dependent cell line HT2, Watson, J.D. "Continuous proliferation of murine antigen specific helper T lymphocytes in culture," 1979, Journal of Experimental Medicine, 150:1510. HT2 proliferation was measured by counting 3H-thymidine incorporation.

Similar experiments were carried out with $(KbP)_2$-EGS and the co-stimulatory antibody OKT3; the results of these experiments are shown in Fig. 12.

IV. Results

Figs. 4 and 5 are graphs showing a dose response curve observed with concentrations of KbP_2-Adipate on anti-CD3 mAb stimulation of H9 cells, as manifested by IL-2 production.

The inventors predict that the observed stimulatory effect with higher KbP_2-Adipate concentrations was due to intermolecular reactions between two different bivalent, dimeric molecules. It is suggested that intermolecular reactions between two different bivalent, dimeric molecules could form via a B-N bond, with the amino group of one divalent molecule binding to a boron atom of a second divalent molecule. This process can continue to form polymers of various lengths. At equilibrium after polymerization, new bivalent compounds with linker spans greater than two times the size of the linker span of one dimeric compound may be formed. For example, the resultant new bivalent

compound, as diagramed in Fig. 7, has lost a binding moiety from each dimer but now has a linker span of greater than twice the original linker span size. Fig. 7 is a diagram showing intermolecular reactions that may occur at higher concentrations of KbP_2-Adipate.

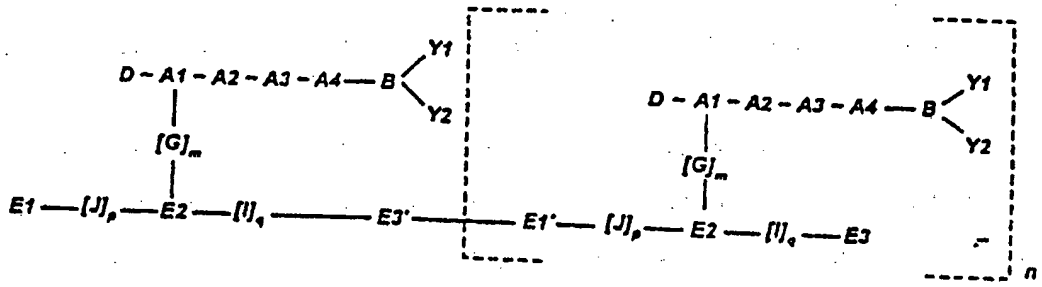
Fig. 12 shows the results of the experiments carried out with KbP_2-EGS, in which the two KbP monomers are linked by the EGS spacer, which is on the order of twice the length of the adipate spacer used to make KbP_2-Adipate homodimer. The results of the experiments with KbP_2-EGS exhibit a pattern analogous to the KbP_2-Adipate results, as Fig. 12 illustrates. For example, there is a co-stimulatory effect with the T cell activating antibody OKT3. Further, the greatest stimulatory effect was observed at low concentrations (between 10^{-6} M and 10^{-12} M) of KbP_2-EGS, and after the peak stimulatory effect (alone or with OKT3), increasing concentrations (10^{-5} M) decreased the activating effect observed.

EXAMPLE 3

HOMOMULTIVALENT POLYMERIC COMPOUNDS DESIGNED TO INDUCE ASSOCIATION BETWEEN T CELL SURFACE RECEPTORS

A variety of different examples of homomultivalent compounds are taught in this example. The standard peptide synthesis methods described above can be used to prepare these compounds.

The general multivalent template has the following structure as shown in Fig. 1R:



wherein D is, independently, selected from the group consisting of NH and NH_2, wherein N represents any isotope of nitrogen, wherein H represents any isotope of hydrogen; "~" is, independently, selected from the group consisting of a single bond and a double bond; B represents, independently, any isotope of boron; A1 is, independently, selected from the

group consisting of a C, a CX moiety and an N, wherein C represents any isotope of carbon, wherein X represents any atom capable of forming a single bond with C; each A2, A3, and A4 are, independently, selected from the group consisting of a CX moiety, a CXZ moiety, a CZ moiety, an NX moiety, and an O, wherein X and Z, independently, are selected from the group consisting of any atom capable of forming a single bond and any atom capable of forming a double bond with C or N and wherein O represents any isotope of oxygen; wherein each Y1 and Y2 are, independently, selected from the group consisting of a hydroxyl moiety and any reactive moiety that converts to a hydroxyl moiety under physiologic conditions; n represents an integer between 1 and 200, inclusive;

wherein E1 and E3 are distinct reactive species in which:

(a) R and R' are the remainder of the molecules not relevant to this reaction;

(b) E1 is attached to R' by a covalent bond which are together designated as E1-R' or R'-E1;

(c) E3 is attached to R by a covalent bond which are together designated as E3-R or R-E3;

(d) R' represents the part of E1-R' not undergoing a chemical reaction;

(e) R represents the part of R-E3 not undergoing a chemical reaction;

(f) E1 undergoes a chemical reaction with E3 to form the product E1'-E3' and a byproduct F, wherein F is selected from the group consisting of $2H^+$ and $2e^-$, H_2O, and any other byproduct;

(g) where H^+ is the cation of any isotope of hydrogen and e^- is an electron;

(h) where H represents any isotope of hydrogen and O represents any isotope of oxygen;

(i) where E1' and E3' are covalently bonded;

(j) E1 does not undergo a chemical reaction with another E1;

(k) E3 does not undergo a chemical reaction with another E3; and

(l) E1 and E3 are selected from the group consisting of a carboxylate, amino, imidazole, sulfhydryl, aldehyde, ester, and any other reactive species;

wherein [J]p, E2, [I]q and [G]m together are a linker moiety, and wherein [G]m, [J]p, and [I]q represent, independently, linker molecules (i) having a molecular weight ranging between about 100 daltons and about 2000 daltons, (ii) having a span ranging from about 20 Å to about 300 Å, and (iii) containing a chain of atoms selected from the group consisting of a combination of C, O, N, S, and Ph atoms, connected by single bonds or by double bonds in a manner that does not violate the laws of chemistry and wherein S represents any isotope of sulfur and Ph represents any isotope of phosphorous; and wherein m, p, and q represent, independently, an integer from 1 to 50, inclusive and wherein E2 is selected from the group consisting of CX, CH, N, PhYZ, PhU, and any other moiety capable of forming covalent bonds with $[J]_p$, $[G]_m$, and $[I]_q$ and wherein:

(a) C is any isotope of carbon;

(b) X is any isotope of any atom capable of forming a single bond with carbon;

(c) H is any isotope of hydrogen;

(d) N is any isotope of nitrogen;

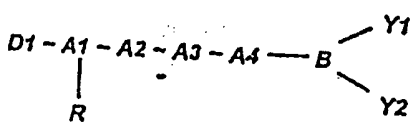
(e) Ph is any isotope of phosphorous;

(f) Y is any isotope of any atom capable of forming a single bond with phosphorous;

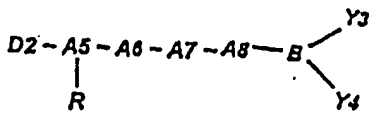
(g) Z is any isotope of any atom capable of forming a single bond with phosphorous; and

(h) U is any isotope of any atom capable of forming a double bond with phosphorous.

Also, the figures shown below represent the binding moiety and R represents the remainder of the molecule in this polymeric compound:



and

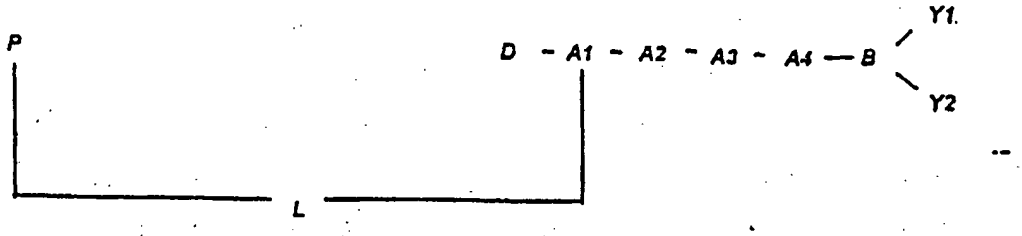


The multivalent compound of this invention can range from a dimer, e.g., n is equal to one (1) or up to about 50-mer, e.g., n is equal to forty-nine (49). When the binding moiety is repeated more than 2 times (a dimer) then the compound would necessarily be a "polymeric" compound with a finite number of repeating binding moieties.

EXAMPLE 4
SYNTHESIS OF HETEROBIVALENT COMPOUNDS DESIGNED TO INDUCE ASSOCIATION BETWEEN CD26 RECEPTOR AND THE T CELL SURFACE RECEPTOR (TCR/CD3)

Constructing heterobivalent (also referred to as heterobidentate or heterodimeric), compounds or agents yields a class of agents capable of inducing an association between CD26 and distinct cell surface receptors. Such heterobifunctional molecules have interesting biological activities and may be useful as drugs.

The general structure for a heterobivalent compound of this invention as shown in Fig. 2A:



wherein D is independently selected from the group consisting of NH and NH_2, wherein N represents any isotope of nitrogen, wherein H represents any isotope of hydrogen; "~", independently, is selected from the group consisting of a single bond and a double bond; B represents, independently, any isotope of boron; A1 is, independently, selected from the group consisting of a C, a CX moiety, and an N, wherein C represents any isotope of carbon, wherein X represents any atom capable of forming a single bond with C; each A2, A3, and A4 are, independently, selected from the group consisting of a CX moiety, a CXZ

moiety, a CZ moiety, an NX moiety, and an O, wherein X and Z, independently, are selected from the group consisting of any atom capable of forming a single bond and any atom capable of forming a double bond with C or N and wherein O represents any isotope of oxygen; wherein each Y1 and Y2 are, independently, selected from the group consisting of a hydroxyl moiety and any reactive moiety that converts to a hydroxyl moiety under physiologic conditions; L represents a linker molecule (i) having a molecular weight ranging between about 100 daltons and about 200 daltons, (ii) having a span ranging from about 20 Å to about 300 Å, and (iii) containing a chain of atoms selected from the group consisting of a combination of C, O, N, S, and Ph atoms, connected by single bonds or by double bonds in a manner that does not violate the laws of chemistry and wherein S represents any isotope of sulfur and Ph represents any isotope of phosphorous; and P represents a peptide ranging from 3 to 30 amino acids having sufficient sequence homology to bind to a naturally occurring receptor. P can be a peptide which selectively binds to CD26 but which does not include a reactive group for forming a covalent bond or complex with the amino acids in the reactive center of the CD26. Such a compound is considered a heterobivalent compound because only one reactive group is present.

Two different heterobivalent compounds (heterodimers) of Lys-boroPro have been constructed and tested. These two compounds are presented as examples only and are not intended to limit the invention. In one heterobivalent compound, Lys-boroPro is linked to the C terminal carboxylate of encephalitogenic myelin proteolipid protein (PLP 139-151; Fig. 2C; see below for discussion). In the second hetero-compound, Lys-boroPro is linked to an antigenic moth cytochrome C peptide (MCC; Fig. 2B; see below for discussion). Both compounds were designed such that association between CD26 and another T cell receptor (TCR/CD3) would be induced. The data presented below demonstrate that both heterobidentate molecules were much more stimulatory than using the peptides alone.

1. Synthesis of Lys-boroPro Linked to Myelin Proteolipid Protein (PLP) Peptide 139-151 to Induce Association Between CD26 Receptor and the T Cell Surface Receptor (TCR/CD3)

Proteolipid protein (PLP) is the major protein of central nervous system myelin. Kuchroo and co-workers have shown that mice immunized with a peptide corresponding to residues 139-151 of PLP, HSLGKWLGHPDKF (SEQ.ID.NO.22) (PLP 139-151) develop acute experimental autoimmune encephalomyelitis (Kuchroo, V.K., et al., Induction of experimental allergic encephalomyelitis by myelin proteolipid-protein specific T cell clones and synthetic peptides, *Pathobiology* 59, 305-312 (1991); Kuchroo, V.K., et al., T-cell receptor alpha chain plays a critical role in antigen-specific suppressor cell function, PNAS (U.S.A.) 88, 8700-8704 (1991); Kuchroo, V.K., et al., Experimental allergic encephalomyelitis mediated by cloned T cells specific for a synthetic peptide of myelin proteolipid protein. Fine specificity and T cell receptor V beta usage, *J. Immunol.* 148, 3776-3782 (1992); Kuchroo, V.K., et al., Cytokines and adhesion molecules contribute to the ability of myelin proteolipid protein-specific T cell clones to mediate experimental allergic encephalomyelitis, *J. Immunol.* 151, 4371-4382 (1993); Kuchroo, V.K., et al., T cell receptor (TCR) usage determines disease susceptibility in experimental autoimmune encephalomyelitis: studies with TCR V beta 8.2 transgenic mice, *Journal of Experimental Medicine* 179, 1659-1664 (1994); and Kuchroo, V.K., et al., A single TCR antagonist peptide inhibits experimental allergic encephalomyelitis mediate by a diverse T cell repertoire, *J. Immunol.* 153, 3326-3336 (1994)). PLP 139-151 also induces the proliferation of T cells in culture. The mechanism involves the T cell receptor (TCR) recognition and binding of this peptide within the context of the major histocompatibility complex (MHC) class II. The MHC is a cluster of genes on human chromosome 6 or mouse chromosome 17 that encodes the MHC molecules. The MHC class I molecules or proteins are the present peptides generated in the cytosol to CD8 T cells. The MHC class II molecules or proteins are the present peptides degraded in cellular vesicles to CD4 T cells. The MHC is the most polymorphic gene cluster known to date in the human genome, having large numbers of alleles at several different loci. Because this polymorphism is usually detected using antibodies or specific T cells, the MHC proteins are often called "major histocompatibility antigens." This allows easy manipulation of the antigenic peptide to convert it from an agonist to an antagonist (Jorgensen, J.L., et al., Molecular components of T-cell recognition, *Annu. Rev. Immunol.* 10, 835-873 91992)).

Systematic amino acid replacement studies have demonstrated that Trp 144 and His 147, shown in bold in the sequence, are necessary for TCR binding while Leu 145 and Pro 148, shown underlined in the sequence above, are necessary for MHC binding.

Crystal structure data on the MHC class II receptor shows that the cleft on top of the molecule which binds the antigenic peptide is open on both sides, which allows longer peptides to be presented by simply permitting them to extent away from the MHC receptor. In contrast, MHC class I receptors only accommodate short peptides from 9 to 12 amino acids and the antigenic peptide ends are not free. The above facts therefore suggest that a bivalent, heterodimer of Lys-boroPro linked to PLP 139-151 could be constructed that would simultaneously bind to the T cell surface receptor (TCR) and CD26 (see Fig. 8) and the MHC II on the antigen presenting cell. Fig. 8 is a diagram showing Lys-boroPro linked to Myelin Proteolipid Protein (PLP) Peptide 139-151.

In the case of PLP, the heterodimer was constructed as HSLGKWLGHPDKFAAAAAA-ϵKbP (SEQ.ID.NO. 23 - ϵKbP) where HSLGKWLGHPDKF (SEQ.ID.NO. 22) was PLP 139-151, AAAAAA (SEQ.ID.NO. 21) was a linker comprised of 6 alanines and ϵKbP was Lysine-boroProline in which the ϵ-amino of Lysine is covalently attached to the -COOH terminus of HSLGKWLGHPDKFAAAAAA (SEQ.ID.NO. 23). The first synthetic step was to order a custom peptide from a synthetic peptide lab. Using long established protocols, the peptide was built from the C-terminus staring with alanine which was immobilized on a resin. Sequentially AAAAA FKPHGLWKGLSH (SEQ.ID.NO.25) were added using protected amino acids. The peptide was then removed from the resin to give a free -COOH terminus which could be reacted to form a peptide bond. The other residues HSLGKWLGHPDKFAAAAA (SEQ.ID.NO. 24) were unreactive owing to protecting groups. Lysine-boroProline in which the α-NH_2 of Lysine was protected, the $B(OH)_2$ of boro Proline was protected with pinanediol, and the ϵ-NH_2 of Lysine was free was coupled to the peptide. The coupling was a peptide bond (-(C=0)-NH-) formed by standard peptide chemistry techniques. The result was then deprotected to yield the final product.

The spacer linker consisting of six consecutive Ala residues was chosen to provide a span sufficient to permit crosslinking (~30Å).

The proliferative effect of PLP-S-KbP on several different T cell clones that recognize PLP 139-151 were tested. T cell clones that recognize an irrelevant epitope, were used as negative controls. The protocol used is described (Kuchroo, V.K., et al., B7-1 and B7-2 Costimulatory Molecules Activate Differently the Th1/Th2 Developmental Pathways: Application to Autoimmune Disease Therapy, *Cell* 80, 707-718 (1995)) and is incorporated herein by reference. Proliferation was measured by thymidine uptake. Table 1 below compares the effect PLP-S-KbP, antigenic PLP 139-151 peptide and non-antigenic PLP 103-116 peptide has on proliferation in five different PLP 139-151 specific T cell clones.

The results show that PLP-S-kbP strongly enhances the proliferative response to PLP 139-151 of all five 139-151 specific clones (see Table 1 below). Enhancement ranges from 100 to over 1000-fold, with respect to the concentration needed to produce a given response. For example, against the first T cell clone listed, 5B8.G8.E6.H12, 0.1 μM of PLP-S-KbP induces almost double the response that a 100-fold higher concentration of PLP 139-151 itself induces, for an enhancement of almost 200-fold. Similarly, nearly a 2000-fold enhancement is induced in the 4E3.B11.D9.H10.H6 cells since 0.1 μM of PLP-S-KbP produces almost double the response a 1000-fold higher concentration of PLP 139-151 itself induces.

These results indicate that a low molecular weight synthetic molecule designed to crosslink CD26 and the TCR, e.g., PLP-S-KbP, strongly enhanced the T cell response to the T cell receptor recognized antigen.

TABLE 1

PROLIFERATIVE EFFECT OF PLP-S-KBP ON SEVERAL

T CELL CLONES SPECIFIC FOR PLP 139-151

Dose (μM)

Antigen	100	10	1	0.1	Clone
PLP-S-KbP	34,716*	53,628	22,022	15,171	5B8.G8.E6.H12
PLP 139-151	41,073	9,176	349	106	
PLP103-116	143	74	226	124	
PLP-S-KbP	31,635	17,516	2,527	681	SPL.C1.H2.F2
PLP 139-151	809	400	107	423	
PLP103-116	150	479	123	238	
PLP-S-KbP	17,608	15,753	8,580	3,688	(4E3.B11.-D9.H10.H6)
PLP139-151	1,932	534	125	318	
PLP103-116	274	224	559	178	
PLP-S-KbP	36,686	26,410	7,738	175	2E5.G10.G5.E5
PLP 139-151	1,506	70	288	101	
PLP103-116	107	60	434	307	
PLP-S-KbP	43,999	35,521	8,202	187	7A5.F10.G11
PLP 139-151	2,324	406	222	117	
PLP 103-116	124	117	314	556	

*^{3}H counts

II. Synthesis of Lys-boroPro Linked to Moth Cytochrome C Peptide (MCC) 94-103 to Induce Association Between CD26 Receptor and the T Cell Surface Receptor (TCR/CD3)

The Moth Cytochrome C (MCC) 94-103 peptide is another antigenic peptide employed in this invention. MCC strongly induces IL-2 production and T cell proliferation when added to cultures of the murine 2B4 T cell hybridoma. The critical residues for binding to the TCR receptor and to the class II MHC are known (Jorgensen, J.L., et al., Molecular components of T-cell recognition, *Annu. Rev. Immunol.* 10, 835-873 (1992)). Because residues critical for MHC and TCR binding are located near the C terminus, Lys-boroPro was coupled to the N terminus of this peptide using a spacer linker of about the same length as used to link Lys-boroPro to the PLP peptide. Standard coupling methodologies were employed. This molecule was designated KbP-S-MCC to signify coupling to the N terminus.

Fig. 9 compares the effect of the bivalent, dimeric KbP-S-MCC molecule and MCC 94-103 itself on IL-2 production in 2B4 cells. The 2B4 T cell hybridoma proliferates in responses to moth cytochrome C peptide 94-103. The peptide 2B4 system is ideal for these studies, since the contest points between MCC 94-103 and the TCR have been determined (Jorgensen, J.L., et al., "Molecular components of T-cell recognition," *Annu. Rev. Immunol.* 10, 835-873 (1992)).

2B4 cells were cultured at 10^{+5}/well with H-2^k APC's and with varying concentrations of KbP-S-MCC and MCC itself. After 24 hours the supernatant was harvested and the IL-2 content determined in a bioassay using HT-2 indicator cells in the same manner as previously described for the PLP peptide assay.

The results demonstrate that coupling Lys-boroPro to MCC strongly enhances the response to the antigenic MCC peptide. Even at the lowest concentration of KbP-S-MCC tested, e.g., 0.4 μM KbP-S-MCC, KbP-S-MCC induced a response at least double that of the maximum response obtained with the MCC peptide alone, which required ~10-fold higher concentration (see Fig. 9).

These results indicate that a low molecular weight synthetic molecule designed to crosslink CD26 and the TCR, e.g., KbP-S-MCC, strongly enhanced the T cell response to the T cell receptor recognized antigen.

III. Synthesis of Lys-boroPro Linked to Other Peptides to Induce Association Between CD26 Receptor and the T cell Surface Receptor (TCR/CD3)

This example describes the synthesis of a preferred binding moiety-reactive group (Lys-boroPro) to certain peptides (e.g., PLP peptides, MCC peptides). It is contemplated that the procedures also can be used for synthesizing compounds in which Lys-boroPro (or an alternative) is coupled to a tumor antigenic peptide or an antigenic peptide of infectious disease. Other heterodimers comprising various derivatives of the PLP or the MCC peptide, which were previously shown to be antagonistic, will be prepared and studies will be performed to determine if linking these peptides to Lys-boroPro enhances their antagonistic activity or transforms them into an agonistic molecule.

Also, tetanus toxoid peptide P2 is of interest because it provides a test system involving human peripheral blood mononuclear cells (PBMC) (Wyse-Coray, T., et al., "Use of antibody/peptides constructs of direct antigenic peptides to T cells: evidence for T cell processing and presentation," *Cellular Immunology*, 139(1):268-73, (1992)). It has been shown that tetanus toxoid peptide P2 peptide induces a response in all HLA haplotypes tested so far (Panina-Bordignon, P., et al., "Universally immunogenic T cell epitopes: Promiscuous binding to human MHC class II and promiscuous recognition by T cells," *Eur. J. Immunol.* 19, 2237-2242 (1989)). Thus, bivalent, heterodimers of Lys-boroPro coupled (linked) to the P2 peptide of tetanus toxoid will be prepared. Different spacer linkers of varying sizes will be tested to determine the optimal length to be used with this peptide. The coupling chemistry needed to produce the bivalent, heterodimer, Lys-boroPro linked to tetanus toxoid peptide P2 peptide, is the same as described above.

Additionally, other molecules capable of inducing the association between CD26 and the T cell surface receptor, e.g., TCR/CD3, can be prepared by coupling Lys-boroPro or other binding moiety to the C terminal or N terminal functional group of different peptides known to bind to the T cell receptor in the context of class II MHC receptors.

Similar protocols as those used to prepare [PLP 139-151]-KbP-S-MCC and KbP-S-MCC, both of which are described above and which involve straightforward standard peptide coupling methodology, can be employed.

EXAMPLE 5
HETEROBIVALENT COMPOUNDS DESIGNED TO INDUCE ASSOCIATION BETWEEN CD26 RECEPTOR AND THE CD4 T CELL SURFACE RECEPTOR

Heterobivalent compounds containing Xaa-boroPro designed to induce association between the CD26 receptor and the CD4 receptor also can be prepared. A molecule known to bind to CD4, e.g., peptides derived from the HIV-1 GP 120 protein, will be coupled to Xaa-boroPro (Ebenbichler, C., et al., "Structure-function relationships of the HIV-1 envelope V3 loop tropism determinant: evidence for two distinct conformations," *Aids* 7, 639-46 (1993); Linsley, P.S., et al., "Effects of anti-gp120 monoclonal antibodies on CD4 receptor binding by the env protein of human immunodeficiency virus type 1," *Journal of Virology* 62, 3695-702 (1988)); Rini, J.M., et al., "Crystal structure of a human immunodeficiency virus type 1 neutralizing antibody, 50.1, in complex with its V3 loop peptide antigen," PNAS (U.S.A.) 90, 6325-9 (1993)).

An Xaa-boroPro molecule, e.g., Lys-boroPro, will be coupled to one of the peptides from the HIV-1 GP 120 protein by using the coupling methodology as described above for coupling Lys-boroPro to the PLP peptide. Of course, any of the other non-boronyl reactive groups described herein can be used in place of the boronyl group to obtain an alternative embodiment.

Different spacer linkers of varying sizes can be evaluated to determine the optimal length for inducing the association between CD26 and CD4.

EXAMPLE 6
HETEROBIVALENT COMPOUNDS DESIGNED TO INDUCE ASSOCIATION BETWEEN CD26 AND OTHER T CELL SURFACE RECEPTORS

Heterobivalent compounds containing Xaa-boroPro designed to induce association between the CD26 receptor and other T cell surface receptors, e.g., granulocyte colony stimulating factor, can be prepared.

For example, cytokine granulocyte colony stimulating factor (G-CSF or granulocyte macrophage colony stimulating factor, GM-CSF), is produced by T cells and macrophages and binds to its own receptor on the T cell surface (the granulocyte colony stimulating factor receptor). A bivalent, heterodimeric form of granulocyte colony stimulating factor could enhance the potency of granulocyte colony stimulating factor by stimulating growth or differentiation or both in cells of myelomonocytic lineage. This compound can be prepared by using standard coupling methodology to couple granulocyte colony stimulating factor to Lys-boroPro. Also, different spacer linkers of varying sizes will be evaluated to determine the optimal length for inducing the association between CD26 receptor and the receptor for colony stimulating factor.

Stem cell factor (c-kit ligand) is essential in stem cell development and binds to the stem cell factor receptor on T cells. In B cell development, CD44 binding probably has no direct signaling function, but instead promotes the binding of a receptor known as c-kit. Lymphoid progenitor cells and early pro-B cells bind to hyaluronic acid on stromal cells via CD44, promoting the binding of their surface c-kit tyrosine kinase to stem cell factor (SCF) on the stromal cell surface, activating the kinase and inducing proliferation. A bivalent, heterodimeric form of stem cell factor, e.g., by coupling to Lys-boroPro, should enhance the potency of SCF.

Lys-boroPro-SCF, heterodimeric compound can be prepared by using methodologies similar to that described above. Different spacer linkers of varying sizes will be tested to determine the optimal length for inducing the association between CD26 receptor and the receptor for stem cell factor.

EXAMPLE 7
SYNTHESIS OF A BIVALENT COMPOUND LINKED TO AN ALL D-AMINO ACID PEPTIDE SYNTHESIZED IN REVERSE TO PREVENT NATURAL PROTEOLYSIS

This example is designed to synthesize a bivalent compound linked to different peptides, whereby the peptides are resistant to natural proteolysis, e.g., Xaa-boroPro coupled to "protected" PLP. In this Example, "protected" means the peptide has been synthesized in the reverse and has a change in chirality.

Retro-Inverso Isomers

Evolution has ensured the almost exclusive occurrence of L-amino acids in naturally occurring proteins. Virtually all proteases therefore cleave peptide bonds between adjacent L-amino acids; thus, artificial proteins or peptides composed of D-amino acids are largely resistant to proteolytic breakdown. This resistance has been attractive to drug designers, but the exclusivity of biological systems for proteins made of L-amino acids means that such proteins cannot interact with the mirror image surface formed by enantiomeric proteins. Thus, an all D-amino acid protein usually has no biological effect or activity.

Linear modified retro-peptide structures have been studied for a long time (Goodman, M., et al., On the Concept of Linear Modified Retro-Peptide Structures, *Accounts of Chemical Research,* 12(1), 1-7 (January, 1979)) and the term "retro-isomer" was designated to include an isomer in which the direction of the sequence is reversed compared with the parent peptide. By "retro-inverso isomer" is meant an isomer of a linear peptide in which the direction of the sequence is reversed and the chirality of each amino acid residue is inverted; thus, there can be no end-group complementarity.

More recently, Jameson et al. reportedly engineered an analogue of the hairpin loop of the CD4 receptor by combining these two properties: reverse synthesis and a change in chirality (Jameson et al., A rationally designed CD4 analogue inhibits experimental allergic encephalomyelitis, *Nature*, 368, 744-746 (1994) and Brady, L. et al., Reflections on a Peptide, *Nature*, 368, 692-693 (1994)). The net result of combining D-enantiomers and reverse synthesis is that the positions of carbonyl and amino groups in each amide bond are

exchanged, while the position of the side-chain groups at each alpha carbon is preserved. Jameson et al. reportedly demonstrated an increase in biological activity for their reverse D-peptide, which contrasts to the limited activity *in vivo* of its conventional all-L enantiomer (owing to its susceptibility to proteolysis).

A partially modified retro-inverso pseudopeptide has been reported for use as a non-natural ligand for the human class I histocompatibility molecule, HLA-A2 (Guichard et al., Partially Modified Retro-Inverso Pseudopeptides as a Non-Natural Ligands for the Human Class I Histocompatibility Molecule HLA-A2, *J. Med. Chem.* 39, 2030-2039 (1996)). The authors report that such non-natural ligands had increased stability and high MHC-binding capacity.

Retroinverso peptides for incorporation in the compounds of the invention (e.g., as P2 peptides in the compound shown in formula I of the Summary of the Invention) are prepared for peptides of known sequence in the following manner. A peptide having a known sequence (e.g., a tumor antigen peptide) is selected as a model peptide for designing and synthesizing a retroinverso peptide analog. The analog is synthesized using D-amino acids by attaching the amino acids in a peptide chain such that the sequence of amino acids in the retroinverso peptide analog is exactly opposite of that in the selected peptide which serves as the model. To illustrate, if the peptide model is a tumor antigen such as the MAGE-1 peptide A1 (formed of L-amino acids) having the sequence, ADPTGHSY, the retroinverso peptide analog of this tumor antigen (formed of D-amino acids) would have the sequence, YSHGTPDA. The procedures for synthesizing a chain of D-amino acids to form the retroinverso peptides are known in the art and are illustrated in the above-noted references.

Since an inherent problem with native peptides is degradation by natural proteases, the heterobivalent or heteromultivalent compounds of this invention will be prepared to include the "retro-inverso isomer" of the desired peptide. Protecting the peptide from natural proteolysis should therefore increase the effectiveness of the specific heterobivalent or heteromultivalent compound.

The protocol given in Example 4, Section 1, for synthesizing PLP 139-151-KbP can be used, with some modifications as necessary, to synthesize the retro-inverso containing

heterodivalent compounds described above. The primary modifications are (1) to synthesize the peptide in reverse and (2) to use D-amino acids for starting materials.

The biological activity of the retro-inverso heterodimer-Lys-boroPro linked to retro-inverso PLP 139-151 can be compared to the control heterodimer-Lys-boroPro linked to conventional PLP 139-151. Activation of T cells can be monitored as described previously in Example 4, Section 1.

A higher biological activity is predicted for the retro-inverso containing heterodimer when compared to the non-retro-inverso containing analog owing to protection from degradation by native proteinases.

EXAMPLE 8
OPTIMAL LINKER MOLECULE DETERMINATION

To determine the optimal linker molecule segment capable of inducing the association of one cell surface CD26 receptor with another cell surface CD26 receptor or other receptor, a series of bivalent, dimeric compounds, with varying length spacer segments (linker molecules), is prepared. A wide assortment of dicarboxylic spacer linker molecules are commercially available (see below for discussion). This includes linker molecules which have various internal heteroatoms and other functional groups, in addition to the terminal carboxylic groups, e.g., ethylene glycobissuccinate.

The corresponding span of the binding sites for monoclonal antibodies varies owing to individual flexibility but is typically about 30 Å to about 100Å. Thus, most linker molecules designed for constructing the homobivalent and heterobivalent compounds of this invention will fall within the general range of about 20 Å to about 300 Å; and more specifically, from about 30 Å to about 100 Å. For example, the spacer linker consisting of six consecutive Ala residues (as used above) was chosen because it was the smallest length spacer believed to be sufficient to crosslink (~30 Å).

Using the linker ethylene glycobissuccinate (instead of adipic acid) provides a bivalent compound with a spacer of about twice the length of the adipoyl moiety. Also, the internal heteroatoms may confer improved water solubility over a straight chain hydrocarbon of similar length.

A. Background on Applicable Linker Molecules

Chemical cross-linkers are valuable tools for scientists and are discussed in numerous books and catalogues, e.g., Pierce Catalog and Handbook, Rockford, Ill. These reagents are used to assist in the determination of near-neighbor relationships in proteins, three-dimensional structures of proteins, enzyme-substrate orientation, solid-phase immobilization, hapten-carrier protein conjugation and molecular associations in cell membranes. They are also useful for preparing antibody-enzyme conjugates, immunotoxins and other labeled protein reagents.

Conjugates consisting of more than two components are difficult to analyze and provide less information on spatial arrangements of protein subunits. The number of components should be kept low or to a minimum. Like many applications, it is necessary to maintain the native structure of the protein complex, so cross-linking agents often employ functional groups that couple to amino acid side chains of peptides. Bifunctional reagents are classified on the basis of the following:

1. Functional groups and chemical specificity
2. Length of cross-bridge
3. Whether the cross-linking groups are similar (homobifunctional) or different (heterobifunctional)
4. Whether the groups react chemically or photochemically
5. Whether the reagent is cleavable
6. Whether the reagent can be radio-labeled or tagged with another label.

Reactive groups that can be targeted using a cross-linker include primary amines, sulfhydryls, carbonyls, carbohydrates and carboxylic acids (to be discussed below). In addition, any reactive group can be coupled nonselectively using a cross-linker such as photoreactive phenyl azides.

Cross-linkers are available with varying lengths of spacer arms or bridges. These bridges connect the two reactive ends. The most apparent attribute of the bridge is its ability to deal with steric considerations of the moieties to be linked. Because steric effects dictate the distance between potential reaction sites for cross-linking, different lengths of bridges are required for the interaction. Normally, a cross-linker with a short spacer arm (4-8 Å) is used and the degree of cross-linking is determined. If this is unsuccessful, a

cross-linker with a longer spacer arm is used. Shorter spacer arms are often used in intramolecular cross-linking studies. Intermolecular cross-linking is favored with a cross-linker containing a longer space arm.

Many factors must be considered to determine optimum cross-linker-to-protein molar ratios. Depending on the application, the degree of conjugation is an important factor. For example, when preparing immunogen conjugates, a high degree of conjugation is normally desired to increase the immunogenicity of the antigen. However, when conjugating to an antibody or an enzyme, a low-to-moderate degree of conjugation may be optimal to ensure that the biological activity of the protein is retained. It is also important to consider the number of reactive groups on the surface of the protein. If there are numerous target groups, a lower cross-linker-to-protein ratio can be used. For a limited number of potential targets, a higher cross-linker-to-protein ratio may be required. This translates into more cross-linker per gram for a small molecular weight protein.

Conformational changes of proteins associated with a particular interaction may also be analyzed by performing cross-linking studies before and after the interaction. A comparison is made by using different arm-length cross-linkers and analyzing the success of conjugation. The use of cross-linkers with different reactive groups and/or spacer arms may be desirable when the conformation of the protein changes such that hindered amino acids become available for cross-linking.

Conjugation reagents contain at least two reactive groups. Homobifunctional cross-linkers can contain at least two identical reactive groups, and heterobifunctional reagents contain two or more different reactive groups. Homobifunctional cross-linkers that couple through amines, sulfhydryls or react non-specifically are available from many commercial sources.

B. Homobifunctional Cross-linkers

Homobifunctional cross-linkers have at least two identical reactive groups and more often are used in a one-step reaction procedure in which the compounds to be coupled are mixed, and the cross-linker is added to the solution. This cross-linking method may result in self-conjugation, intramolecular cross-linking and/or polymerization.

1. Primary Amine-Reactive Groups

There are two major types of homobifunctional imidoesters and homobifunctional N-hydroxysuccinimidyl (NHS) esters. Commercially available homobifunctional imidoesters range in size from about 8 Å to about 11.9 Å. Commercially available homobifunctional N-hydroxysuccinimidyl esters range in size from about 6.2 Å to about 16.1 Å.

Because primary amines are commonly found in proteins, homobifunctional NHS ester cross-linkers are the most commonly used conjugation reagents. Both yield stable derivatives.

2. Sulfhydryl-Reactive Group

Maleimides, alkyl and aryl halides, α-haloacyls and pyridyl disulfides are thiol reactive groups. These reagents react faster with sulfhydryls, making them thiol-selective. Maleimides, alkyl and aryl halides, and α-haloacyls react with sulfhydryls to form thiol either bonds. Pyridyl disulfides react with sulfhydryls to produce mixed disulfides. The pyridyl disulfide product is cleavable.

3. Nonselective Group

A nonselective homobifunctional is useful for conjugating functional groups, such as hydroxyls for which specific cross-linkers are not available.

An example of a nonselective homobifunctional cross-linker is BASED (Product #21564 Pierce Co.). This cross-linker has a long spacer arm and 2 aromatic rings which makes it very hydrophilic with a limited solubility in aqueous systems. This cross-linker also has a large diffusion capacity and should permeate membranes before conjugation initiates.

C. Heterobifunctional Cross-linkers

Heterobifunctional cross-linkers possess two or more different reactive groups that allow for sequential conjugations with specific groups of proteins, minimizing undesirable polymerization or self-conjugation. Heterobifunctional reagents frequently are used when modification of amines is problematic. Amines may sometimes be found at the active sites

of macromolecules, and the modification of these may lead to the loss of activity. Other moieties such as sulfhydryls, carboxyls, phenols and carbohydrates may be more appropriate targets. A two-step strategy allows for the coupling of a protein that can tolerate the modification of its amines to a protein with other accessible groups.

A variety of heterobifunctional cross-linkers, each combining different attributes for successful conjugation are commercially available. The majority of commercially available heterobifunctional cross-linkers contain an amine-reactive functional group. Cross-linkers that are amine-reactive at one end and sulfhydryl-reactive at the other end are quite common. Commercially available heterofunctional cross-linkers range in size from about 0 Å (EDC, Pierce Co.) to about 15.6 Å.

When using heterobifunctional reagents, the most labile group should be reacted first to ensure effective cross-linking and avoid unwanted polymerization. Most heterobifunctional cross-linkers are sulfhydryl-reactive NHS-esters. The sulfhydryl reactive groups are usually maleimides, pyridyl disulfides and α-haloacetyls. Carbodiimides are carboxyl and amine reactive.

Heterobifunctional reagents, in which the reactivity can be controlled and that contain one group that is spontaneously non-reactive, have distinct advantages. This allows for specific attachment of the labile group first; the second reaction can then be initiated when appropriate. A selection of heterobifunctional reagents that contain at least one photoaffinity group are commercially available. This selection includes iodinatable and cleavable reagents that react nonspecifically at the azido group and with amines, sulfhydryls, carbohydrates and carbonyls. Often a bifunctional photoactivatable cross-linker has a better chance of forming a covalent cross-link than a bifunctional chemically reactive cross-linker. The high reactivity of the photochemical reagent allows for formation of a conjugate that may not be possible with a group-specific reagent. However, the yield resulting from a photoreactive cross-linker is low, and yields of less than 10% should be considered acceptable.

D. Reactivities of Different Chemical Groups

1. Imidoester Cross-linkers

Imidoester homobifunctional cross-linkers were among the first used to immobilize proteins onto solid-phase supports. They were used extensively for the study of protein structure and molecular associations in membranes. Although these cross-linkers are still used in protein subunit studies and solid-phase immobilization, they have been steadily replaced by the more stable, more efficient homobifunctional NHS-ester cross-linkers. Homobifunctional imidoesters maintain the net electronic charge on protein after cross-linking. Spacer arm lengths range from about 8.6 Å to about 11.9 Å. Imidoester cross-linkers react rapidly with amines at alkaline pH, but they have short half lives.

Imidoesters are also very useful for protein-protein cross-links. These cross-linkers can penetrate cell membranes and cross-link proteins within the membrane to study membrane composition, structure and protein-protein and protein-lipid interactions. Imidoesters are also useful for oligomer formation. For example, cross-linking proteins to form oligomers may reveal if a bivalent, dimeric or trimeric form of the protein is responsible for activity.

2. N-Hydroxysuccinimide-Esters (NHS-esters)

NHS-esters yield stable products upon reaction with primary or secondary amines. Coupling is efficient at physiological pH, NHS-ester cross-linkers are also more stable in solution than their imidate counterparts. Homobifunctional NHS-ester conjugations are commonly used to cross-link amine-containing proteins in either one-step or two-step reactions.

Primary amines are the principle targets for NHS-esters. Accessible α-amine groups present on the N-termini of proteins react with NHS-esters and form amides. However, because α-amines on a protein are not always available, the reaction with side chains of amino acids become important. While five amino acids have nitrogen in their side chains, only the ϵ-amines react significantly with NHS-esters. A covalent amide bond is formed when the NHS-ester cross-linking agent reacts with primary amines, releasing N-hydroxysuccinimide.

3. Coupling through Sulfhydryl Groups

Coupling through sulfhydryl groups is advantageous because it can be site-directed, yield cleavable products and allow for sequential coupling. A protein in a complex mixture can be specifically labeled if it is the only one with a free sulfhydryl group on its surface.

a. Maleimides

The maleimide group is specific for sulfhydryl groups when the pH of the reaction mixture is kept between pH 6.5 and 7.5. At pH 7, the reaction of the maleimides with sulfhydryls is 1000-fold faster than with amines. Maleimides do not react with tyrosines, histidines or methionines.

b. Haloacetyls

The most common used α-Haloacetyls react with sulfhydryl groups at physiological pH. The reaction of the iodoacetyl group with a sulfhydryl proceeds by nucleophilic substitution of iodine, with a thiol producing a stable thioether linkage. Selectivity for sulfhydryl groups is ensured by using only a slight excess of the iodoacetyl group over the number of sulfhydryl groups at pH 8.3. In the absence of free sulfhydryls, or if a gross excess of iodoacetyl group is used over the number of sulfhydryls, the iodoacetyl group can react with other amino acids.

c. Pyridyl Disulfides

Pyridyl disulfides react with sulfhydryls groups to form a disulfide bond. Pyridine-2-thione is released as a by-product of this reaction. These reagents can be used as cross-linkers and to introduce sulfhydryl groups into proteins.

4. Coupling Through Carboxyl Groups: Carbodiimides

Carbodiimides couple carboxyls to primary amines or hydrazides, resulting in formation of amide or hydrazone bonds. Carbodiimides are unlike other conjugation reactions in that no cross-bridge is formed between the molecules being coupled. Carboxy termini of proteins can be targeted, as well as glutamic and aspartic acid side chains. In the presence of excess cross-linker, polymerization is likely to occur because proteins contain carboxyls and amines. No cross-bridge is formed, and the amide bond is the same as a peptide bond, so reversal of the cross-linking is impossible without destruction of the

protein. EDC (Pierce Co.) reacts with carbocyclic acid group and activates the carboxyl group, allowing it to be coupled to the amino group (R_4NH_2) in the reaction mixture.

5. Nonselective Labeling: Arylazides

A photoaffinity reagent is a compound that is chemically inert but becomes reactive when exposed to ultraviolet or visible light. Arylazides are photoaffinity reagents that are photolyzed at wavelengths between 250-460 nm, forming a reactive aryl nitrene. The aryl nitrene reacts nonselectively to form a covalent bond. Reducing agents must be used with caution because they can reduce the azido group.

6. Nonselective Labeling

a. Arginine Specific Cross-linkers

Glyoxals are useful compounds for targeting the guanidinyl portion of arginine residues. Glyoxals will target arginines at mildly alkaline pH. There is some cross-reactivity (the greatest at higher pH) with lysines.

b. Carbonyl Specific Cross-Linkers

Carbonyls (aldehydes and ketones) react with amines and hydrazides at pH 5-7. The reaction with hydrazides is faster than with amines, making this useful for site-specific cross-linking. Carbonyls do not readily exist in proteins; however, mild oxidation of sugar moieties using sodium metaperiodate will convert vicinal hydroxyls to aldehydes or ketones.

7. Applications for Use of Cross-linkers

a. Cell Surface Cross-linking

To ensure cell-surface specific cross-linking for identification of surface receptors or their ligands, it is best to use membrane-impermeable cross-linkers. In the past, researchers used water-insoluble cross-linkers and carefully controlled the amount of cross-linker and the cross-linking duration. This prevented penetration of the membrane by the cross-linker and the cross-linking duration. This prevented penetration of the membrane by the cross-linker and subsequent reaction with membrane proteins. Many references cite the use of membrane-permeable cross-linkers for cell surface cross-linking.

b. Subunit Cross-linking and Protein Structural Studies

Cross-linkers can be used to study the structure and composition of proteins in biological samples. Some proteins are difficult to study because they exist in different conformations under varying pH or salt conditions. One way to avoid conformational changes is to cross-link the subunits together. Amine-, carboxyl- or sulfydryl-reactive reagents are employed for identification of particular amino acids or for the determination of the number, location and size of subunits in a protein. Short-to-medium spacer arm cross-linkers are selected when intramolecular cross-linking is performed. If the spacer arm is too long, intermolecular cross-linking can occur. Carbodiimides that result in no spacer arm, along with short length conjugating reagents are commercially available.

c. Subunit Cross-linkers and Protein Structural Studies

Cross-linkers are widely used for identification of near-neighbor protein relationships, ligand-receptor identification and interactions, and enzyme substrate orientations. The cross-linkers chosen for these applications are usually longer than those used for subunit cross-linking. Homobifunctional, amine-reactive NHS-esters or imidates and heterobifunctional, amine-reactive photoactivatable phenyl azides are the most commonly-used cross-linkers for these procedures. Occasionally, a sulfhydryl- and amine-reactive cross-linker may be employed if one of the two proteins or molecules is known to contain sulfhydryls. Cleavable or noncleavable cross-linkers typically are used. Because the distances between two molecules are not always known, the optimum length of the spacer arm of the cross-linker may be determined by the use of a panel of similar cross-linkers with different lengths. NHS-ester, phenylazides are very useful for this type of cross-linking because they usually result in some successful, if not efficient, cross-linking.

Cross-linkers can be used to determine whether a particular protein is located on the surface or integral to the membrane. These studies are possible because water-soluble cross-linkers are membrane-impermeable, while water-insoluble cross-linkers are membrane permeable.

d. Cell Membrane Structural Studies

Cell membrane structural studies require reagents of varying hydrophobicity to determine the location and the environment within a cell's lipid bilayer. Fluorescent tags are used to locate proteins, lipids or other molecules inside and outside the membrane.

Various cross-linkers with differing spacer arm lengths can be used to cross-link proteins to associated molecules within the membrane to determine the distance between molecules. Successful cross-linking with shorter cross-linkers is a strong indication that two molecules are interacting in some manner. Failure to obtain cross-linking with a panel of shorter cross-linkers, while obtaining conjugation with the use of longer reagents, generally indicates that the molecules are located in the same part of the membrane but are not interacting. Homobifunctional NHS-esters, imidates or heterobifunctional NHS-esters, photoactivatable, phenyl azides are commonly used for these procedures.

8. Immunotoxins

Specific antibodies can be covalently linked to toxic molecules and then used to target antigens on cells. Often these antibodies are specific for tumor associated antigens. Immunotoxins are brought into the cell by surface antigens and, once internalized, they proceed to kill the cell by ribosome inactivation or other means. The type of cross-linker used to make an immunotoxin can affect its ability to locate and kill the appropriate cells. For immunotoxins to be effective, the conjugate must be stable *in vivo*. In addition, once the immunotoxin reaches its target, it is important that the antibody be separable from the toxin to allow the toxin to kill the cell. Thio-cleavable, disulfide-containing conjugates have been shown to be more cytotoxic to tumor cells than noncleavable conjugates of ricin A immunotoxins. Cells are able to break the disulfide bond in the cross-linker, allowing the release of the toxin within the targeted cell.

9. Carrier Protein-Hapten/Peptide/Polypeptide Conjugates for Use as Immunogens

Companies, e.g., Pierce Co., offer products in this area of immunological research. Easy-to-use kits are available for coupling ligands using several different chemistries. There are many cross-linkers used for the production of these conjugates, and the best choice is dependent on the reactive groups present on the hapten and the ability of the

hapten-carrier conjugate to function successfully as an immunogen after its injection. Carbodiimides are good choices for producing peptide carrier conjugates because both proteins and peptides usually contain several carboxyls and primary amines.

Other heterobifunctional cross-linkers can also be used to make immunogen conjugates. Often peptides are synthesized with terminal cysteines to allow for their attachment to supports or to carrier proteins through a part of the molecule that is not important for activity or recognition. Sulfhydryl-reactive, heterobifunctional cross-linkers can be coupled to carrier proteins through their other functional group and then can be linked to peptides through terminal cysteines. This method can be very efficient and yield an immunogen that is capable of eliciting a good response upon injection.

10. Solid-Phase Immobilization

Proteins, peptides and other molecules can be immobilized on solid-phase matrices for use as affinity supports or for sample analysis. The matrices may be agarose, beaded polymers, polystyrene plates or balls, porous glass or glass slides, and nitrocellulose or other membrane materials. Some supports can be activated for direct coupling to a ligand. Other supports are made with nucleophiles or other functional groups that can be linked to proteins or other ligands using cross-linkers.

Immobilization of the compounds of the invention to solid-supports can be accomplished using routine coupling chemistries. In general, the compounds of the invention are immobilized by including in the compounds an accessible first functional group (e.g., an alcohol group) and contacting the compound with a solid-support containing a complementary second functional group (e.g., carboxyl groups) under conditions and for a period of time sufficient to permit the first and the second functional groups to react with one another to form a covalent bond (e.g., ester bond). By "accessible" in reference to a functional group, it is meant that the functional group is in a form which is reactive and is not sterically precluded from reacting with the solid-support.

The functional groups for immobilizing the compounds of the invention to a solid-support can be introduced into the peptide binding moieties or the linker portions of these compounds. For example, amino acids that include functional groups in their side chains (e.g., aspartate, glutamate, cysteine residues) can be incorporated into the peptide binding

moiety during synthesis and positioned at a sufficient distance from the reactive group which binds to the target protein to avoid unwanted steric hindrance by the solid-support in the reaction between the compound and its target protein. Alternatively, the compounds of the invention can be immobilized via a functional group in the linker molecule to a solid-support. Thus, for example, the linkers which are used in this aspect of the invention can include, in addition to the first and the second linker reactive groups for binding to the first and the second peptide binding moieties, a further functional group for binding to the solid support. Trifunctional molecules of this type are illustrated in Example 11. To prevent side reactions, it is preferred that the linker reactive groups that are used for coupling the linker molecule to the peptide binding moieties be different from functional groups that are used for coupling the linker to the solid-support. Such functional groups can be introduced into the linker molecules at any time during or after the synthesis of these molecules. Thus, in general, the same types of functional groups, protection/deprotection reactions and reagents, and reaction conditions that are established in the art for using linker molecules to couple, e.g., proteins or peptides to one another or to solid supports can be used for immobilizing the compounds of the invention to a solid support.

11. Protein-Protein Conjugates

One of the most widely used applications for cross-linkers is the production of protein-protein conjugates. Biological assays require methods for detection, and one of the most common methods for quantitation of results is to conjugate an enzyme, fluorophore or other molecule to a protein that has affinity for one of the components in the biological system being studied. Antibody-enzyme conjugates (primary or secondary antibodies) are among the most common protein-protein conjugates used. Secondary antibodies are relatively inexpensive and are commercially available.

Listed below is a representative sampling of commercially available cross-linkers, e.g., from Pierce Catalog and Handbook, Rockford, Ill. The table also identifies which group the linker is reactive towards, e.g., sulfhydryls, carboxyls.

REACTIVITY OF COMMERCIALLY AVAILABLE CROSS-LINKERS

	Reactive Towards					Cleavable By			
Double-Agent Cross-linker Acronym	$-NH_2$ Aminos	-SH Sulfhydryls	Carbohydrates	Non-selective (Photo-reactive)	-COOH Carboxyls	Thiols	Base	Periodate	Hydroxylamine
ABH			X	X					
ANB-NOS	X			X					
APDP		X		X		X			
APG				X					
ASIB		X		X					
ASBA				X	X				
BASED				X		X			
BS'	X								
BMH		X							
BSOCOES	X						X		
DFDNB	X								
DMA	X								
DMP	X								
DMS	X								
DPDPB		X				X			
DSG	X								
DSP	X					X			

	Reactive Towards					Cleavable By			
Double-Agent Cross-linker Acronym	-NH_2 Amines	-SH Sulfhydryls	Carbohydrates	Non-selective (Photo-reactive)	-COOH Carboxyls	Thiols	Base	Periodate	Hydroxylamine
DSS	X								
DST	X							X	
DTBP	X					X			
DTSSP	X					X			
EDC	X				X				
EGS	X								X
GMBS	X	X							
HSAB	X			X					
LC-SPDP	X	X							
MBS	X	X				X			
H_2C_2H		X	X						
MPBH		X	X						
NHS-ASA	X			X					
PDPH		X	X			X			
PNP-DTP	X			X					
SADP	X			X		X			
SAED	X			X		X			
SAND	X			X		X			

	Reactive Towards					Cleavable By			
Double-Agent Cross-linker Acronym	-NH, Amines	-SH Sulfhydryls	Carbohydrates	Non-selective (Photo-reactive)	-COOH Carboxyls	Thiols	Base	Periodate	Hydroxylamine
SANPAH	X			X					
SASD	X			X		X			
SDBP	X								
SIAB	X	X							
SMCC	X	X							
SMBP	X	X							
SMPT	X	X							
SPDP	X	X				X			
Sulfo-BSOCOES	X						X		
Sulfo-DST	X							X	
Sulfo-EGS	X								X
Sulfo-GMBS	X	X							
Sulfo-HSAB	X			X					
Sulfo-LC-SPDP	X	X				X			
Sulfo-MBS	X	X							
Sulfo-NHS-ASA	X			X					
Sulfo-NHS-LC-ASA	X			X					
Sulfo-SADP	X			X		X			

	Reactive Towards					Cleavable By			
Double-Agent Cross-linker Acronym	-NH, Amines	-SH Sulfhydryls	Carbohydrates	Non-selective (Photo-reactive)	-COOH Carboxyls	Thiols	Base	Periodate	Hydroxylamine
Sulfo-SAMCA	X			X					
Sulfo-SANPAH	X			X					
Sulfo-SAPB	X			X					
Sulfo-SIAB	X	X							
Sulfo-SMCC	X	X							
Sulfo-SMBP	X	X							
Sulfo-LC-SMPT	X	X							

EXAMPLE 9
PREVENTION OF CYCLIZATION BY DESIGNING A COMPOUND CONTAINING AN OLEFIN GROUP

In this Example, Xaa-boroPro analogs containing an olefin group, e.g., a fluoroolefin will be constructed to prevent cyclization and increase biological activity. The biological activity of his olefin containing compound will be tested by the methods described previously in Example 2, section III, e.g., comparing the production of IL-2 using an olefin containing compound versus using a compound that does not contain an olefin.

1. Identification of Active (Open) and Inactive (Cyclic) Species of Monomeric Compounds as Related to Inhibitory Activity of Soluble CD26 (DP IV)

The inventors have previously shown that synthetic diastereomeric monomeric compounds, e.g., L-Ala-D,L-boroPro and L-Pro-D,L-boroPro, were potent inhibitors of the catalytic activity of soluble DP IV (CD26). They also encountered a problem because these monomeric inhibitors lost some of their inhibitory activity rapidly in aqueous solution at a pH of about neutral. For example, Ala-boroPro lost DP IV inhibitory activity with a half-life of about 5 minutes and pro-boroPro lost activity with a half life of about one hour. It was determined that the inhibitors did not undergo degradation in aqueous solutions of neutral pH and higher, but did undergo a cyclization reaction.

In aqueous solution at all pH values, the inhibitors exist as a slowly equilibrating mixture of two conformations: an open chain structure which is inhibitory (active species), and a cyclic structure which is non-inhibitory (inactive species). See Fig. 10 which is a diagram showing the structures of the open and cyclized forms of Xaa-boroPro inhibitors (conformational equilibrium of Xaa-boroProline inhibitors). The open, active, inhibitory chain species is favored at low pH while the cyclized structure is favored at high pH. The reaction is fully reversible: the open chain becomes predominate at low pH. The open chain to cyclic species reaction involves a trans to cis isomerization of the proline and the formation of a new N-B bond. The cyclized structure is the boron analog of a diketopiperazine, a product often seen in peptide chemistry. Cyclization liberates one equivalent of H+ thereby explaining the requirement for base in the cyclization reaction and

acid in the opening reaction. The cyclic structure is quite stable in aqueous solutions of high pH.

Prolonged incubation at high pH never leads to the complete disappearance of DP IV inhibitory activity for any of the Xaa-boroPro compounds examined. This observation was the first evidence that the active inhibitor was in a conformation equilibrium with a non-inhibitory species rather than undergoing an irreversible inactivation. The half life for the reformation of the open chain species from the cyclic structure is surprisingly low. Thus, the loss of inhibitory activity in aqueous solution was due to a pH dependent conformational equilibrium rather than a degradation reaction.

The fact that the inhibitory activity does not go to zero for any of the Xaa-boroPro inhibitors, even after prolonged incubation, together with the fact that the reverse reaction, i.e., cyclic to open chain is slow, suggested that it should be possible to measure the equilibrium constant for the conformation equilibrium by measuring the apparent Ki at equilibrium and comparing it with the true Ki.

It has been demonstrated that the ratio of [cyclic] : [open] forms, at neutral pH, is 156:1 for Pro-boroPro and 1130:1 for Val-boroPro (W.G. Gutheil and W.W. Bachovchin, Separation of L-Pro-DL-boroPro into Its Component Diastereomers and Kinetic Analysis of Their Inhibition of Depeptidyl Peptidase IV. A New Method for the Analysis of Slow, Tight-Binding Inhibition, *Biochemistry* 32, 8723-8731 (1993)). This means that less than 1% Pro-boroPro and less than 0.1% of Val-boroPro exists as the open chain, inhibitory species, at equilibrium at pH 7.0. Nevertheless, under these conditions the inhibitors behave as though they had Ki's of 2.5 nM and 1.8 nM respectively. This apparent Ki of the "fully inactivated" species is still substantially better than, (~1000-fold) that of other inhibitors of DP IV thus far reported.

2. Background Information on Olefin Containing Compounds

Previously, fluoroolefin peptide isosteres have been used as tools for controlling peptide conformations (Boros, L.G., et al Fluoroolefin Peptide Isosteres - Tools for Controlling Peptide Conformations, *Tetrahedron Letters*, 35(33), 6033-6036 (1994)). Fluoroolefin containing dipeptide isosteres have also been shown to be effective inhibitors of dipeptidyl peptidase IV (CD26) (Welch, J.T. et al Fluoroolefin Containing Dipeptide

Isosteres as Inhibitors of Dipeptidyl Peptidase IV (CD26), *Tetrahedron*, 52(1), 291-304 (1995)).

3. Prevent Cyclization to Increase Biological Activity of Bivalent or Multivalent Compound

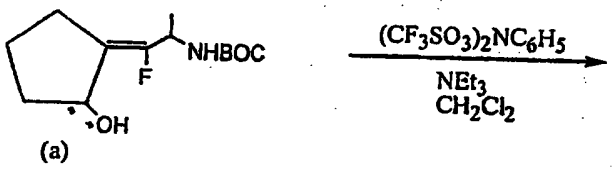
The inventors predict that biological bioavailability (biological function) for the compounds taught in this invention could be significantly increased (approximately 100-1000 times) by preventing peptide conformational changes, e.g., intermolecular cyclization, by constructing a bivalent or multivalent compound containing an olefin group (a carbon atom double bonded to another carbon atom; see Fig. 11D), e.g., fluoroolefin. Figs. 11A-11D are diagrams of different examples of bivalent compounds containing an olefin group and are not meant to limit the scope of the invention. Fig. 11D is a fluoroolefin isostere of Xaa-boroProline. A fluoroolefin mimics a peptide bond but prevents cis trans isomerization and therefore prevents cyclization. Thus, if cyclization can be blocked, the inventors predict that the bioavailability of the compounds taught herein can be increased by approximately 100 - 1000 fold.

The methods used to synthesize fluoroolefins are described below. See also, Livia G. Boros, et al "Fluoroolefin peptide isosteres - tools for controlling peptide conformations," *Tetrahedron Letters*, Vol. 35, No. 33, pp. 6033 and 6036, 1994; and John T. Welch and Jian Lin, "Fluoroolefin containing dipeptide isosteres as inhibitors of dipeptidyl peptides IV (CD26)", *Tetrahedron*, Vol. 52, No. 1, pp. 291-304, 1996 for alternative procedures that can be adapted to prepare the compounds of the invention.

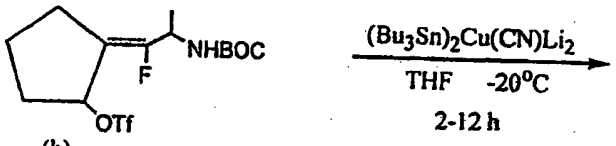
The procedures for forming a particularly preferred fluorolefin compound of the invention are provided. However, it is contemplated that any of the compounds of the invention can be designed and constructed to incorporate an olefin group using these procedures. Accordingly, it will be appreciated that the procedures can be used to prepare olefin-containing boronyl compounds of the invention in which the olefin is unsubstituted or substituted with a halogen other than fluorine. Further, it will be appreciated that although a compound having a boronyl reactive group is illustrated, a reactive group other than a boronyl group can be prepared by using an analog of compound (a) in which an alternative reactive group is substituted for BCl_3 in the reaction. The synthesis of

alternative non-boronyl group, olefin-containing compounds can be performed using the synthesis procedures identifed above in reference to the Ferring published PCT application.

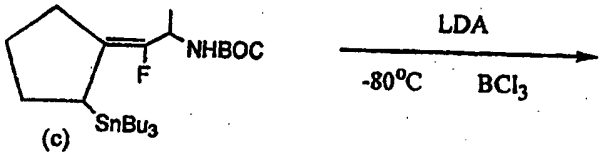
The synthesis protocol involves three reactions. In the first reaction, compound (a) is converted to compound (b) using the reaction conditions set forth in Welch, J. et al., Tetrahedron 52:291-304 (1996). In the second reaction, compound (b) is converted to compound (c) using the reaction conditions set forth in Tilley, J.W. et al., J. Med. Chem. 34:1125-1136 (1991). In the third reaction, compound (c) is converted to the fluorolefin-derivative compound using the reaction conditions set forth in Lipshultz, B.H. et al., J. Org. Chem. 54:4975 (1989).



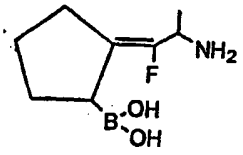
(a)



(b)



(c)



The fluoroolefin analogs of Xaa-boroPros will be compared to Xaa-boroPros by measuring the potency of inhibition of CD26 proteinase activity. The immunodulatory effects of these fluoroolefin containing analogs will be evaluated by in vivo experiments using animal models and in vitro experiments using cell culture. The cell culture experiments will monitor cytokine production by cells of lymphoid origin, proliferation of cells of lymphoid origin, or both.

EXAMPLE 10
SYNTHESIS OF FLUORESCENTLY LABELLED MONOMERIC DERIVATIVES OF LYS-BOROPRO

Fluorescently labelled derivatives of monomeric LysboroPro can be prepared and used to determine if monomeric Lys-boroPro induces internalization of CD26 into cells. Fluorescence microscopy can be used to monitor intercellular trafficking of CD26.

One labelling approach is directly couple the side chain amino group of monomeric Z-Lys-boroPro-diester to a functional group on a fluorescent molecule such as the isothiocyanate of fluorescein isothiocynate (FITC). Lys-boroPro can be linked to FITC either directly or through a spacer linker molecule.

Another labelling approach is to link the side chain amino group of Lys-boroPro to biotin, again either directly or through a spacer and then use the avidin, strepavidinbiotin systems for detection.

Avidin is a glycoprotein found in the egg white and tissues of birds, reptiles and amphibia. This protein contains four identical subunits having a combined molecular mass of 67,000-68,000 daltons. Each subunit binds one molecule of biotin, and studies have shown that tryptophan and lysine are involved in the binding site for biotin. The sequencing of the subunit indicates it consists of 128 amino acids. Avidin has an isoelectric point of 10-10.5 and is very soluble in water and salt solutions. Avidin is stable over a wide range of pHs and temperatures. Extensive chemical modification has little effect on the activity of avidin, making it useful for detection and protein purification.

Streptavidin is another biotin-binding protein, and it is isolated from *Streptomyces avidinii*. The molecular weight of streptavidin is about 60,000. Unlike avidin, streptavidin

has no carbohydrate and has an acidic isoelectric point of 5. Streptavidin is much less soluble in water than avidin and can be crystallized from water or 505 isopropanol.

Advantages of this approach over the above approach include: (1) the biotin-avidin system is well developed, well described and has been successfully utilized for the type of work proposed herein in many other systems; (2) improved flexibility because a large number of reagents are commercially available for use in this system: (3) improved sensitivity because this system provides for amplification of the signal from the biotinylated moiety (e.g., the biotinylated Lys-boroPro can be rendered fluorescent by reaction with either avidin (or streptavidin) conjugated either to a fluorochrome such as FITC or conjugated to an enzyme such as horseradish peroxidase). Avidin-FITC conjugate has many FITC groups per avidin molecule compared to only one of the first approach described above. The avidin-enzyme conjugate approach provides a large amplification owing to the many molecules of substrate converted to detectable product per avidin molecule.

N-hydroxysuccinmide biotin (NHS-biotin, available from Pierce Company) reacts readily with free primary amines to form a biotin conjugate linked with a peptide bond. First, NHS-biotin, and NHS-LC-biotin (which has a 22.4 Å spacer arm to separate biotin from the amino group of the molecule to which it is to be conjugated) is linked to the side chain amino group of Lysine of Lys-boroPro, and these molecules are characterized as to their ability to inhibit DP IV and their ability to serve as a detection tool for Lys-boroPro-protein complexes using the avidin detection systems.

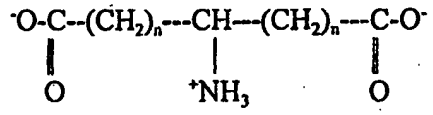
Each of the compounds prepared as described above can be purified to homogeneity using HPLC and its identity can be confirmed by NMR spectroscopy, amino acid composition, or mass spectroscopy as deemed necessary.

EXAMPLE 11
SYNTHESIS OF FLUORESCENTLY LABELLED BIVALENT AND MULTIVALENT DERIVATIVES OF LYS-boroPRO

Fluorescently labelled dimeric and multimeric derivatives containing preferred P^1R^1 monomers (e.g., Lys-boroPro) can be prepared and used to determine if bivalent and

trivalent derivatives of, e.g., Lys-boroPro, can induce aggregation and internalization of cells surface CD26.

Producing such molecules can be accomplished in several ways. One way is to use a trifunctional molecule of the type illustrated below. With appropriate chemical methodology, the carboxylate groups can be coupled to Lys-boroPro molecules while the amino group can be coupled to an FITC molecule, or other fluorophore, or more preferably, to biotin via a suitable length spacer. Trifunctional molecules of the type shown below are available or can be synthesized relatively easily.



Trimeric forms of Lys-boroPro also are possible through straightforward extension of the approach described above for the bivalent forms. Another approach takes advantage of the multimeric nature of the biotin-avidin system. Avidin has four subunits, each of which has a biotin binding site for biotin. If fluorescent trimeric inhibitors are needed, biotin can be coupled to Lys-boroPro through a suitable length spacer (the NHS-LC-biotin should have an adequate length spacer arm 22.4 Å). The effect of various amounts of avidin, both on T cell activation and on cell surface CD26 aggregation can be determined using FITC-conjugated avidin. This approach likely yields a mixture of divalent, trivalent, and tetravalent inhibitors.

The compounds prepared as described above can be purified by HPLC and their structure can be confirmed by NMR spectroscopy, amino acid composition, or mass spectroscopy as necessary.

EXAMPLE 12
SYNTHESIS OF LYS-BOROPRO LINKED TO INSOLUBLE SUPPORTS

Lys-BoroPro linked to insoluble supports may be useful for three reasons. The first is for determining the effect of such solid phase immobilization of Lys-boroPro on T cell proliferation for comparison with analogous experiments with solid phase immobilized

anti-CD26 mAbs. Solid phase immobilized Lys-boroPro derivatives should induce aggregation of cell surface CD26, but differ from soluble, multimeric inhibitors in that they should prevent internalization, or at least internalization of inhibitor bound CD26. The second use is for determining if Lys-boroPro binds to proteins other than CD26 to an appreciable extent. The third use is to produce an affinity column for producing purified CD26 from various sources.

The bi and multivalent compounds of the invention, as well as P^1R^1 monomers (e.g., Lys-boroPro), can be immobilized on solid supports in many ways, each with certain advantages. Initially, solid state immobilized avidin together with biotinylated Lys-boroPro can be tested because this approach offers substantial flexibility. Solid state immobilized avidin, e.g., linked to agarose, is commercially available (Pierce Chemical Co.) with avidin and can be obtained in both multimeric and in monomeric forms. The monomeric form is designed to allow for the removal and recovery of biotinylated proteins from the resin and therefore may be preferred for the second and third purposes described above. There are, however, other ways to provide for the removal and recovery of biotinylated Lys-boroPro protein conjugates. For example, (i) high concentration of biotin may compete with and displace the biotinylated Lys-boroPro from the solid state immobilized avidin, (ii) free Lys-boroPro may displace the biotinylated inhibitor from the proteins, (iii) a biotin derivative with a cleavable group in the spacer arm can be used in preparing the biotinylated Lys-boroPro, (iv) lowering the pH to ~4.0 will dramatically lower the affinity of Lys-boroPro for the active site of DP IV and thus should allow elution of the protein from the resin leaving behind biotinylated Lys-boroPro attached to avidin (G.R. Flentke, et al., Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhibitors to examine the role of DP-IV in T-cell function, PNAS (USA) 88, 1556-1559 (1991); Bachovchin, W.W. et al., Inhibition of IgA1 proteinases from Neisseria gonorrhoea and Hemophilus influenzae by peptide prolyl boronic acids, J. of Biol. Chem. 265, 3738-43 (1990)). Diols, such as pinanediol or pinacol, can be added to the elution buffer to bind to and thus tie up the hydroxyl groups on the boronyl group.

Each of the compounds prepared as described above can be purified to homogeneity using HPLC and its identity can be confirmed by NMR spectroscopy, amino acid composition, or mass spectroscopy as deemed necessary.

EXAMPLE 13
CD26 (DP IV) AFFINITY STUDIES

Since the purification of CD26 has remained a major problem, employing an affinity column as described below should be of substantial benefit. The fact that the linked homobivalent, dimeric molecule, KbP_2-Adipate, was shown to be a potent inhibitor at lower concentrations, e.g., 10^{-10}M, (See Example 1, I, B(1) above)) suggests that an immobilized compound of the invention can be used for affinity purification of CD26. Derivatizing the ϵ-amino group of the Lys-boroPro, without sacrificing affinity, should help in developing an affinity column specific for the purification of CD26 from various sources, e.g., cell lines transfected with CD26 (DP IV) genes.

EXAMPLE 14
MEASURING STANDARD CD26 (DP IV) ACTIVITY

Assays to measure CD26 (DP IV) activity can be performed on both the homobivalent compounds, e.g., Lys-boroPro coupled to another, and the heterobivalent compounds, e.g., Lys-boroPro coupled to a peptide specific for a T cell surface receptor, e.g., moth cytochrome C peptide. Methods for quantitatively measuring the interaction of small peptidomimetic inhibitors with CD26 or DP IV, as well as for the interaction of CD26 with larger ligands, e.g., the HIV Tat protein, have been developed (W.G. Gutheil and W.W. Bachovchin. Separation of L-Pro-DL-boroPro into Its Component Diastereomers and Kinetic Analysis of Their Inhibition of Dipeptidyl Peptidase IV. A New Method for the Analysis of Slow, Tight-Binding Inhibition, *Biochemistry* 32, 8723-8731 (1993); Gutheil, W.G., and W., B.W. Kinlsq, A Matlab Program for Fitting Kinetics Data with Numerically Integrated Rate Equations and Its Application to the Analysis of Slow, Tight Binding Data, *Analytical Biochemistry 223,* 13-20 (1994); Gutheil, W.G., et al., HIV-1 Tat Binds to DP IV (CD26): A possible Mechanism for Tat's Immunosuppressive

Activity, *Proc. Natl. Acad. Sci. U.S.A.* 91, 6594-6598 (1994)). These methods use the chromatogenic substrate Ala-Pro-p-nitroanilide (AppNA) and fluorescent substrate Ala-Pro-7-amino-4-trifluoromethyl coumarin (AP-AFC). AppNA and AP-AFC are commercially available (e.g., Enzyme Systems Products, Dublin, CA).

EXAMPLE 15
IMMUNOLOGICAL STUDIES ON HOMOBIVALENT AND HETEROBIVALENT COMPOUNDS

1. T-cell Functional Assays

The ability of bivalent Lys-boroPro molecules designed to induce association of cell surface CD26 can be measured in an antigen specific T cell response as previously described (G.R. Flentke, et al., "Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhibitors to examine the role of DP-IV in T-cell function," PNAS (USA) 88, 1556-1559 (1991); M. Subramanyam, et al., "Mechanism of HIV-1 Tat induced inhibition of antigen-specific T cell responsiveness," J. Immunol. 150, 2544-2553 (1993)).

Briefly, in the human system, peripheral blood mononuclear cells (PBMC) are cultured with suboptimal doses of anti-CD3 mAbs, in the presence or absence of the CD26 associating agents. Alternatively, a recall antigen response is measured to a suboptimal concentration of tetanus toxoid or candida antigen, plus or minus the CD26 associating agents (M. Subramanyam, et al., "Mechanism of HIV-1 Tat Induced inhibition of antigen-specific T cell responsiveness," J. Immunol. 150, 2544-2553 (1993)).

In the murine system, the cytochrome C system is used for measuring the response in the 2B4 T cell hybridoma (G.R. Flentke, et al., "Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhibitors to examine the role of DP-IV in T-cell function," *PNAS (USA)* 88, 1556-1559 (1991)). Cellular activation is measured by the production of IL-2 by the T cell hybridoma, and the level of IL-2 produced is quantified in a biological assay, using HT-2 indicator cells.

2. Internalization of CD26/Drug Delivery

Because antibody mediated crosslinking of CD26 induces internalization, it is likely that homobivalent compounds of Lys-boroPro also have this biological activity. Receptor internalization can be determined by employing FITC labelled bivalent Lys-boroPro and performing flow cytometric analysis (N.H. Dang, Y. Torimoto, K. Sugita, J.F. Daley, P. Schow, C. Prado, S.F. Schlossman, and C. Morimoto. Cell surface modulation of CD26 by anti-1F7 monoclonal antibody: Analysis of surface expression and human T cell activation, *Journal of Immunology* 145, 3963-3971 (1990)). To compare cell membrane versus cytoplasmic staining, the cells are analyzed (i) with intact membranes, allowing only cell surface staining, and (ii) after permeabilization of the membrane with saponin, which allows the antibody to cross the membrane, although the membrane stays structurally intact (de Caestecker, M.P., Telfer, B.A., Hutchinson, I.V., and Ballardie, F.W., "The detection of intercytoplasmic interleukin 1α, interleukin 1β and tumour necrosis factor α expression in human monocytes using two colour immunofluorescence flow cytometry," *J. Immunol. Methods* 154, 11-20 (1992). Once it is demonstrated that the CD26 molecule becomes internalized after incubation with a bivalent or multivalent derivative of Lys-boroPro, a larger molecule is coupled to the bivalent molecule, preferentially an enzyme that can be easily detected, e.g., luciferase, alkaline phosphate or β-galactosidase. Expression of these proteins can be measured using a cellular extract. Kits are commercially available for this determination (e.g., Novacasatra Laboratories Ltd., Newcastle upon Tyne, UK). Furthermore, mAbs are commercially available (e.g., Southern Biotech, Birmingham Alabama) for these enzymes, enabling detection of protein expression in single cells by flow cytometry. As above, saponin can be used to permeabilize the cells, enabling entry of the mAbs. This method is very sensitive and allows the simultaneous analysis of other cell surface or cytoplasmic proteins.

Uses and Advantages

As described herein, the invention provides a number of uses and advantages. The low molecular weight, synthetic bivalent or multivalent compounds of the invention are designed to induce the association between association-activated receptors and, therefore, offer considerable potential for regulating biological systems. Thus, this new class of

biologically active compounds, or compositions thereof, are useful for inducing the association between association-activated receptors on human T cells and for the treatment of a wide variety of T-cell mediated diseases in animals; for example, autoimmune disease.

Generally, these bivalent or multivalent homo- or hetero-compounds or compositions thereof are useful as immune response modulating therapeutics (1) to treat disease conditions characterized by immunosuppression, e.g., AIDS or AIDS-related complex, other virally or environmentally induced conditions and certain congenital immune deficiencies; (2) to increase an immune function which has been impaired by the use of immunosuppressive drugs, (3) to treat systemic lupus erythematosis, rheumatoid arthritis, and multiple sclerosis.

For example, the compounds of this invention can be used to deliver a member of the superantigen family to stimulate T cells. Superantigens comprise a class of disease-associated, immunostimulatory molecules that bind class II MHC molecules and stimulate large numbers of T cells (Jardetzky, T.S., et al. "Three Dimensional Structure of a Human Class II Histocompatiblity Molecule Complexd with Superantigen", *Nature* 368, 711-718 (April, 1994)). Members of the superantigen family include toxins from *S. aureus* and other bacteria, as well as viral superantigens from mouse mammary tumor virus. The toxicity of the bacterial superantigens is thought to be mediated by their potent T-cell stimulating activities, leading to lymphokine release, respiratory distress and shock. Superantigens have also been implicated in rabies, rheumatoid arthiritis, and mouse and human AIDS.

These bivalent or multivalent homo- or hetero-compounds or compositions thereof can be used to stimulate the growth of hemapopoietic cells in culture. Such cells can be transplanted into mammals, e.g., humans, to stengthen or boost the hematopoietic, immune system, or both. These compounds also can be used to treat patients suffering from disease or from deficiency of hematopoietic cells such as AIDS patients, patients undergoing chemotherapy and/or patients under radiotherapy for hematological or other cancers, and patients undergoing bone marrow transplants.

When administered to mammals, e.g., humans, the compounds of the invention may enhance the ability of the immune system to regenerate cells that are immunosuppressed, e.g., CD4 and T cells. Thus, the bivalent compounds of this invention, can be administered

to mammals, e.g., humans, in an effective amount alone or in combination with a pharmaceutically acceptable carrier, excipient, or diluent, in unit dosage form. Conventional pharmaceutical practice can be employed to provide suitable formulations or compositions to administer these compounds to patients suffering from immunosuppression or an immune deficiency or presymptomatic of AIDS. Any appropriate route of administration may be employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intrcapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Therapeutic formulations may be in the form of liquid solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found in, for example, "Remington's Pharmaceutical Sciences." Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene polyoxyproplyene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for these bivalent compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems or liposomes. Formulations of inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel.

These bivalent homo- or heterbivalent compounds or compositions thereof can be used to control the T cell activation process and thus be used to prevent unwanted immune responses.

The homobivalent compounds designed to induce the association between one CD26 T cell receptor and another CD26 T cell receptor can be useful in stimulating the proliferation of CD4+ cells. These compounds could, therefore, help restore CD4+ cells numbers in immunosuppressed patients, e.g., AIDS patients, and thereby reverse the decline in immune function.

The homobivalent compounds or compositions thereof (CD26 T cell receptor associated with another CD26 T cell receptor) of the invention can be used to enhance recall-antigen specific immune responses. Lymphocytes from most HIV-infected individuals exhibit a qualitative defect in their ability to respond to recall antigens (A.S. Fauci. The human immunodeficiency virus: infectivity and mechanisms of pathogenesis. *Science* 239, 617-722 (1988)). This defect is exhibited early after infection and long before CD4+ T cell number decline. Accordingly, it is believed that these compounds will be useful in treating AIDS. Thus, the stimulatory activities should improve lymphocyte function in HIV infected individuals by ameliorating the defective recall antigen responses which show up early after infection, and by improving CD4+T cell numbers.

Since the compounds of the invention and, in particular, the compounds of claim 1 have high affinity and specificity for CD26, they can be useful for the selective delivery of other therapeutic agents to, and into CD4+ T cells. Thus, the compounds of the invention can be used to deliver pharmacological agents inside CD4+ cells that normally are unable to penetrate such cells. For example, many highly potent inhibitors of the HIV protease have been developed, which despite their high affinities for the HIV proteinase, are limited in blocking HIV *in vivo* owing to their inability to get inside the CD4+ cells. These HIV proteinases hibitors inhibitors can be linked to the spacer arm of a bivalent CD26 ligand and delivered into CD26+ lymphocytes, which are the cells the virus primarily invades. Even if a drug is capable of entering CD4+ cells, the compounds of the invention can be used to concentrate the drug in CD26 cells, thereby maximizing the desired pharmacological activity while minimizing unwanted toxic side effects on other cells. This delivery vehicle therefore provides a mechanism to prevent or reduce AZT resistance by providing higher concentrations of AZT in CD4+ cells and lower concentrations elsewhere. Thus, the CD26 internalization activity of the compounds disclosed herein can be used to provide a vehicle for delivering and concentrating other therapeutic agents into CD4+T cells.

The heterobivalent compounds designed to induce the association between a CD26 T cell receptor and a different T cell receptor, e.g., the T cell receptor TCR/CD3, can be useful in stimulating a cell-mediated immune response against specific antigens. Heterobivalent compounds or compositions thereof comprising a CD26 inhibitor and an

antigenic peptide stimulate a cellular immune response against an antigenic peptide as compared to an antibody-mediated immune response. This stimulation of cell-mediated response against specific antigens can be useful in patients with AIDS because these patients have high concentrations of anti-HIV antibodies. Thus, the CD26-TCR association-inducing activity can be useful for stimulating a cell mediated immune response to specific antigens. This biological activity should prove especially useful in vaccine development, particularly for AIDS because a cellular or T_H1 immune response is the appropriate response for HIV-1 and is apparently lacking in AIDS patients.

Such heterobivalent compounds can be useful in the development and manufacture of peptide-based vaccines and therapeutic agents for the treatment of allergies and autoimmune diseases.

Because the response stimulated is cell mediated and specific for the antigen chosen, such bifunctional agents can be useful in the development of vaccines for modulating a cell mediated immune response, especially for the development of a vaccine for treating AIDS.

The heterobivalent compounds designed to induce the association between a CD26 T cell receptor and a different T cell receptor, e.g., CD4, can be useful in activating T cell function. This heterobivalent compound or compositions thereof can be used to selectively deliver agents to CD4+ CD26+ cells, or they can be useful in blocking HIV entry into CD4+ cells.

The heterobivalent compounds or compositions thereof, e.g., as shown in claim 17, can be used to deliver a toxin, e.g., ricin A immunotoxin or AZT, within a CD26+ T cell. A toxin of choice can be coupled to a dimeric compound having high affinity for the CD26 T cell surface receptor. Also, a toxin can be coupled to a peptide having specificity for a specific T cell receptor. Either way, once the dimeric compound associates or binds to the CD26 T cell surface receptor, the toxin is internalized within the T cell, thereby delivering this toxin to a T cell.

The compounds or compositions thereof can be administered alone or in combination with one another, or in combination with other therapeutic agents. For example, treatment with one of the bivalent compounds can be combined with more traditional therapies for treating disorders of the immune.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically-acceptable amounts and in pharmaceutically-acceptably compositions. Such preparations may routinely contain salt, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically-acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically-acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically-acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts. The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as: benzalkonium chloride; chlorobutanol; parabens and thimerosal. Compositions suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the crosslinking compound, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intramuscular, etc.

administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

A variety of administration routes are available for treating a subject. The particular mode of delivery selected will depend, of course, upon the particular crosslinking compound selected, the severity of the condition being treated and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, rectal, topical, nasal, interdermal, or parenteral routes. Such modes of administration also include obtaining T cells or bone marrow cells, stem cells or early lineage progenitor cells from a patient and contacting the isolated cells with the crosslinking compounds of the invention ex vivo, followed by reintroducing the treated cells to the patient. The treated cells can be reintroduced to the patient in any manner known in the art for administering viable cells.

As used herein, the term "parenteral" includes subcutaneous, intravenous, intramuscular, or infusion. Intravenous or intramuscular routes are not particularly suitable for long-term therapy and prophylaxis. They could, however, be preferred in emergency situations. Oral administration will be preferred for prophylactic and other treatment because of the convenience to the patient as well as the dosing schedule.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. The methods include the step of bringing the crosslinking compounds of the invention into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the crosslinking compounds into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the crosslinking compound of the invention. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the crosslinking compounds described above, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono- di- and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which the crosslinking compound is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,667,014, 4,748,034 and 5,239,660 and (b) diffusional systems in which an active component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,832,253, and 3,854,480. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be particularly suitable for treatment of chronic conditions. Long-term release, as used herein, means that the implant is constructed and arranged to delivery therapeutic levels of the active ingredient for at least 10 days, and preferably 60 days. Long-term sustained release

implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The crosslinking compounds described herein are administered in effective amounts. An effective amount is a dosage of the crosslinking compound sufficient to provide a medically desirable result. The effective amount will vary with the particular condition being treated, the age and physical condition of the subject being treated, the severity of the condition, the duration of the treatment, the nature of the concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. For example, an effective amount for stimulating T cell activation would be an amount sufficient to increase to a statistically significant extent T cell activation as for example, measured by proliferation or by increased T cell activity. An effective amount for stimulating a desired immune response also can be measured, for example, by determining a change in the immune function in a subject (e.g., increased B cell response, increased cytotoxic T cell response, stimulation of bone marrow proliferation, or an ability to slow, halt, or prevent an infection or cancer). An effective amount for treating an autoimmune disorder or allergic disorder would be that amount sufficient to lessen or inhibit altogether the immune or allergic response associated with the disorder so as to slow or halt the development of or the progression of the disorder. Thus, it will be understood that the crosslinking compounds of the invention can be used to treat an autoimmune disorder (e.g., transplant rejection) prophylactically in subjects at risk of developing an immune response (e.g., recipients prior to transplant). As used in the claims, "inhibit" embraces all of the foregoing. Likewise, an effective amount for treating an immune system disorder is that amount which can slow or halt altogether the symptoms associated with the immune system disorder so as to prevent the disorder, slow its progression, or halt the progression of the immune system disorder. It is preferred

generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment.

Generally, doses of active compounds will be from about 0.001mg/kg per day to 1000 mg/kg per day. It is expected that doses range of 0.01 to 100 mg/kg will be suitable, preferably orally and in one or several administrations per day. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of compounds.

Other embodiments are within the following claims.

What is claimed is:

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Trustees of Tufts College

(ii) TITLE OF THE INVENTION: MULTIVALENT COMPOUNDS FOR CROSSLINKING RECEPTORS AND USES THEREOF

(iii) NUMBER OF SEQUENCES: 27

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Wolf, Greenfield & Sacks, P.C.
(B) STREET: 600 Atlantic Avenue
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: U.S.A.
(F) ZIP: 02210

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 28-JUN-1997
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: 08/671,756
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(A) APPLICATION NUMBER: 08/837,305
(B) FILING DATE: 11-APR-1997

(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: I0254/7007

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-720-3500
(B) TELEFAX: 617-720-2441

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu Ala Asp Pro Thr Gly His Ser Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Ala Tyr Gly Glu Pro Arg Lys Leu
1 5

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Glu Val Asp Pro Ile Gly His Leu Tyr

1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Leu Leu Ala Val Leu Tyr Cys Leu
1 5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Tyr Met Asn Gly Thr Met Ser Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Tyr Leu Glu Pro Gly Pro Val Thr Ala
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Leu Asp Gly Thr Ala Thr Leu Arg Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Ala Gly Ile Gly Ile Leu Thr Val
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ser Ser Ser Thr Leu Cys Thr Ser Lys Ala Asp Lys Ser Ser Gly Asn
1 5 10 15
Gln Gly Gly Asn Gly Val Phe Ile Val Val Asn Ala Trp Tyr Ser
20 25 30

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Ser Glu Asp Leu Thr Ala Gly Tyr Cys Lys Cys Phe Glu Glu Phe Val
 1               5                  10                  15
Leu Ala Ser Arg Cys Lys
            20
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Glu Gln Arg Gln Gly Ile Lys Val Gln Leu Ile Leu Phe Ile Leu Arg
 1               5                  10                  15
Ala Leu Met Ile Asn Thr Ser Ser Ser Asn His Ile Leu Asp Ser Arg
            20                  25                  30
Asn Val Phe Leu His Thr Gly His Gly Glu Pro Met Val Gln Lys Gln
        35                  40                  45
Ile Glu Trp Val Leu Ile Met Glu Leu Ile Lys Met
    50                  55                  60
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
His Lys Ala Val Phe Arg Ser Glu Ile Ser Leu Gln Lys Trp Cys Ser
```

1 5 10 15
Asp Thr Gln Lys Ser Thr
20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Asp Ser Phe Glu Ser Val Arg Leu Pro Ala Pro Phe Arg Val Asn His
1 5 10 15
Ala Val Glu Trp
20

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ile Ile Ser Pro Val Ile Phe Gln Ile Ala Leu Asp Lys Pro Cys His
1 5 10 15
Gln Ala Glu Val Lys His Leu His His Leu Leu Lys Gln Leu Lys Pro
20 25 30
Ser Glu Lys Tyr Leu Lys Ile Lys His Leu Leu Leu Lys Arg Glu Arg
35 40 45
Val Asp Leu Ser Lys Leu Gln
50 55

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 37 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Arg Ser Lys Thr Leu His His Leu Leu Lys Gln Leu Lys Pro Ser Glu
1 5 10 15
Lys Tyr Leu Lys Ile Lys His Leu Leu Leu Lys Arg Glu Arg Val Asp
20 25 30
Leu Ser Lys Leu Gln
35

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Pro Pro Gln Thr Gly Glu Lys Tyr Leu Lys Ile Lys His Leu Leu Leu
1 5 10 15
Lys Arg Glu Arg Val Asp Leu Ser Lys Leu Gln
20 25

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Asp Ala Asp Thr Tyr Tyr Ile Leu Pro Arg Lys Val Leu Gln Met Asp
1 5 10 15
Phe Leu Val His Pro Ala
20

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Asp Thr Leu Leu Leu Leu Pro Arg Lys Val Leu Gln Met Asp Phe Leu
1 5 10 15
Val His Pro Ala
20

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Leu His Phe Ala Ser Arg Trp Ile Phe Leu Phe Ile Gln Pro Glu Cys
1 5 10 15
Ser Glu Pro Arg
20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Gln Asp Leu Thr Met Lys Tyr Gln Ile Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ala Ala Ala Ala Ala Ala
1 5

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

His Ser Leu Gly Lys Trp Leu Gly His Pro Asp Lys Phe
1 5 10

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His Ser Leu Gly Lys Trp Leu Gly His Pro Asp Lys Phe Ala Ala Ala
1 5 10 15
Ala Ala Ala

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

His Ser Leu Gly Lys Trp Leu Gly His Pro Asp Lys Phe Ala Ala Ala
1 5 10 15
Ala Ala

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Ala Ala Ala Ala Ala Phe Lys Asp Pro His Gly Leu Trp Lys Gly Leu
1 5 10 15
Ser His

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Ala Asp Pro Thr Gly His Ser Tyr
1 5

(2) INFORMATION FOR SEQ ID NO:27:

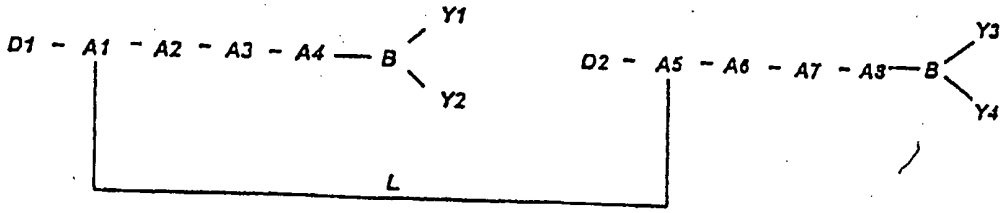
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Tyr Ser His Gly Thr Pro Asp Ala
1 5

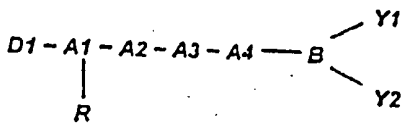
CLAIMS

1. A compound, having the structure

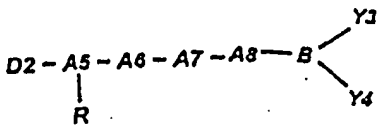


wherein D1 and D2, independently, are selected from the group consisting of NH and NH2, wherein N represents any isotope of nitrogen, wherein H represents any isotope of hydrogen; "~"is, independently, selected from the group consisting of a single bond and a double bond; B represents, independently, any isotope of boron; A1 and A5 are, independently, selected from a group consisting of a C, a CX moiety and an N, wherein C represents any isotope of carbon, wherein X represents any atom capable of forming a single bond with C: each A2, A3, A4, A6, A7 and A8 are, independently, selected from a group consisting of a CX moiety, a CXZ moiety, a CZ moiety, an NX moiety, and an O, wherein X and Z, are, independently, selected from the group consisting of any atom capable of forming a single bond and any atom capable of forming a double bond with C or N and wherein O represents any isotope of oxygen; wherein each Y1, Y2, Y3 and Y4 are, independently, selected from the group consisting of a hydroxyl moiety and any reactive moiety that converts to a hydroxyl moiety under physiological conditions; and L represents a linker molecule (i) having a molecular weight ranging between about 100 daltons and about 2000 daltons, (ii) having a span ranging from about 20 Å to about 300 Å, and (iii) containing a chain of atoms selected from the group consisting of a combination of C, O, N, S, and Ph atoms, connected by single bonds or by double bonds in a manner that does not violate the laws of chemistry and wherein S represents any isotope of sulfur and Ph represents any isotope of phosphorous.

2. The compound of claim 1 wherein the following structures



and



represent, independently, a binding moiety, wherein R represents the remainder of the molecule.

3. The compound of claim 2 wherein there are 4 atoms positioned between the group consisting of D1 and D2 and B of the binding moiety.

4. The compound of claim 2 wherein the binding moiety is in an L-configuration.

5. The compound of claim 2 wherein Y1, Y2, Y3 and Y4 are hydroxyl groups.

6. The compound of claim 1 wherein the A4 bonded to the B is in the L-configuration and the A5 bonded to the B is in the L-configuration.

7. The compound of claim 2 wherein the binding moiety is an L-amino acid residue conjugated to B, a boron molecule.

8. The compound of claim 2 wherein the binding moiety is selected from the group consisting of L-Lys-L-boroPro and a derivative of L-Lys-L-boroPro.

9. The compound of claim 1 wherein the linker molecule contains a functional group selected from the group consisting of a carboxylate group, an amino group, a sulfhydryl group, an imidazole group, an alkene group, an acyl halogen group, and CH_2X, wherein X represents a halogen.

10. The compound of claim 1 wherein the linker molecule is further defined as having the following structure:

wherein [G] contains atoms selected from the group consisting of a carbon, nitrogen, oxygen, hydrogen and a sulfur atom; [J] is selected from the group consisting of a CH_2 molecule, a chain of carbon atoms, a chain of nitrogen atoms, and a chain of oxygen atoms; and m, p, and q represent an integer from 1 to 50, inclusive.

11. The compound of claim 10 wherein [G] is an R group selected from the group consisting of L-amino acid residues selected from the group consisting of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine and D-amino acid residues selected from the group consisting of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine.

12. The compound of claim 1 wherein the linker molecule is selected from the group consisting of hexanedioic acid (adipic acid), EGS, 1, 4-diaminobutane, 1, 4-dithiobutane, dithiothreitol, lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine.

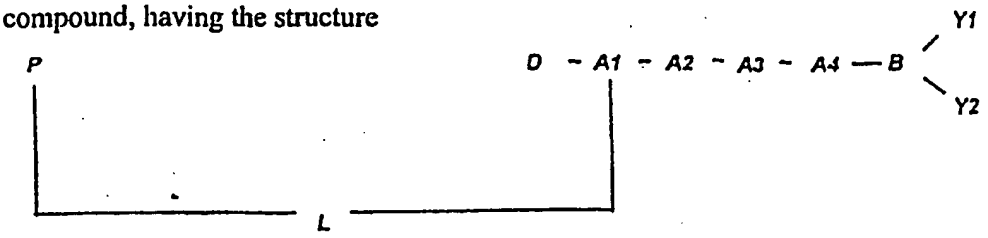
13. The compound of claim 1 wherein the linker molecule contains at least two amino groups when the binding moieties contain glutamic acid residues.

14. The compound of claim 1 wherein the linker molecule contains at least two amino groups when the binding moieties contain aspartic acid residues.

15. The compound of claim 1 wherein the linker molecule contains at least two sulfhydryl groups when the binding moieties contain cysteine residues.

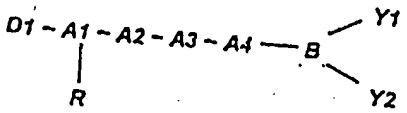
16. The compound of claim 1 wherein the linker molecule span ranges from about 30 Å to about 100 Å.

17. A compound, having the structure

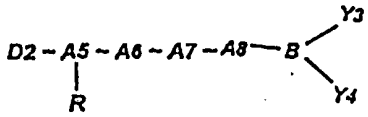


wherein D is independently selected from the group consisting of NH and NH_2, wherein N represents any isotope of nitrogen, wherein H represents any isotope of hydrogen; "~"is, independently, selected from the group consisting of a single bond and a double bond; B represents, independently, any isotope of boron; A1 is, independently, selected from a group consisting of a C, a CX moiety and an N, wherein C represents any isotope of carbon, wherein X represents any atom capable of forming a single bond with C; each A2, A3 and A4 are, independently, selected from a group consisting of a CX moiety, a CXZ moiety, a CZ moiety, an NX moiety, and an O, wherein X and Z, independently, are selected from the group consisting of any atom capable of forming a single bond and any atom capable of forming a double bond with C or N and wherein O represents any isotope of oxygen; wherein each Y1 and Y2 are, independently, selected from the group consisting of a hydroxyl moiety and any reactive moiety that converts to a hydroxyl moiety under physiological conditions; L represents a linker molecule (i) having a molecular weight ranging between about 100 daltons and about 2000 daltons, (ii) having a span ranging from about 20 Å to about 300 Å, and (iii) containing a chain of atoms selected from the group consisting of a combination of C, O, N, S, and Ph atoms, connected by single bonds or by double bonds in a manner that does not violate the laws of chemistry and wherein S represents an isotope of sulfur and Ph represents any isotope of phosphorous; and P represents a peptide ranging from 3 to 30 amino acids having sufficient sequence homology to bind to a naturally occurring receptor.

18. The compound of claim 17 wherein the following structures



and



represent, independently, a binding moiety, wherein R represents the remainder of the molecule.

19. The compound of claim 18 wherein there are 4 atoms positioned between D and B of the binding moiety.

20. The compound of claim 18 wherein the binding moiety is in an L-configuration.

21. The compound of claim 17 wherein Y1 and Y2 are hydroxyl groups.

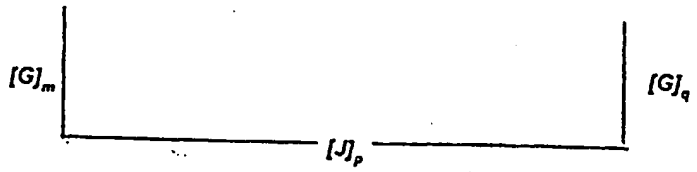
22. The compound of claim 17 wherein the A4 bonded to the B is in the L-configuration.

23. The compound of claim 18 wherein the binding moiety is an L-amino acid residue conjugated to B, a boron molecule.

24. The compound of claim 18 wherein the binding moiety is selected from the group consisting of L-Lys-L-boroPro and a derivative of L-Lys-L-boroPro.

25. The compound of claim 17 wherein the linker molecule contains a functional group selected from the group consisting of a carboxylate group, an amino group, a sulfhydryl group, an imidazole group, an alkene group, an acyl halogen group, and CH_2X, wherein X represents a halogen.

26. The compound of claim 17 wherein the linker molecule is further defined as having the following:



wherein [G] contains atoms selected from the group consisting of a carbon, nitrogen, oxygen, hydrogen and a sulfur atom; [J] is selected from the group consisting of a CH_2 molecule, a chain of carbon atoms, a chain of nitrogen atoms, and a chain of oxygen atoms; and m, p and q represent an integer from 1 to 50, inclusive.

27. The compound of claim 26 wherein [G] is an R group selected from the group consisting of L-amino acid residues selected from the group consisting of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine and D-amino acid residues selected from the group consisting of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine and asparagine.

28. The compound of claim 17 wherein the linker molecule is selected from the group consisting of adipic acid, between 2 and 15 consecutive amino acid residues, 1, 4-diaminobutane, 1,4-dithiobutane, and dithiothreitol.

29. The compound of claim 17 wherein the linker molecule span ranges from about 30 Å to about 100 Å.

30. The compound of claim 17 wherein the peptide ranges from about 7 to 25 amino acids.

31. The compound of claim 17 wherein the peptide is selected from the group consisting of:

a) a peptide derived from a human myelin protein.

b) a moth cytochrome C peptide;

c) a tetanus toxin;

d) a HIV-1 GP 120 peptide;

e) myelin basic protein peptide;

f) a tumor antigenic peptide; and

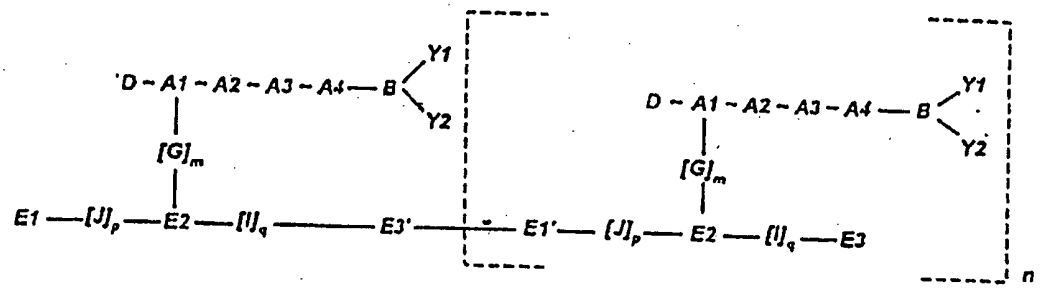
g) antigenic peptides of infectious agents.

32. The compound of claim 31 wherein the human Myelin protein peptide is selected from the group consisting of a myelin basic protein; a mylene proteolipid protein, and a myelin associated glycoprotein, wherein the myelin proteolipid protein peptide is selected from the group consisting of PLP peptide 139-151 and PLP peptide 190-209, wherein the Moth cytochrome C peptide is peptide MCC 94-103, wherein the myelin basic protein peptide is MBP peptide 1-11, and wherein the tetanus toxin peptide is selected from the group consisting of tetanus toxoid peptide and P2 tetanus toxoid peptide.

33. The compound of claim 17 wherein the naturally occurring receptor is a T cell surface receptor or a B cell surface receptor.

34. The compound of claim 33 wherein the cell surface receptor is selected from the group consisting of TCR/CD3, CD2, CD4, CD8, CD10, CD26, CD28, CD40, CD45, B7.1 and B7.2.

35. A compound, having the structure



wherein D is independently selected from the group consisting of NH and NH_2, wherein N represents any isotope of nitrogen, wherein H represents any isotope of hydrogen; "~" is, independently, selected from the group consisting of a single bond and a double bond; B represents, independently, any isotope of boron; A1 is, independently, selected from a group consisting of a C, a CX moiety and an N, wherein C represents any isotope of carbon, wherein X represents any atom capable of forming a single bond with C: each A2, A3 and A4 are, independently, selected from a group consisting of a CX moiety, a CXZ moiety, a CZ moiety, an NX moiety, and an O, wherein X and Z, independently, are selected from the group consisting of any atom capable of forming a single bond and any atom capable of forming a double bond with C or N and wherein O represents any isotope of oxygen; wherein each Y1 and Y2 are, independently, selected from the group consisting of a hydroxyl moiety and any reactive moiety that converts to a hydroxyl moiety under physiological conditions; n represents an integer between 1 and 200, inclusive;

wherein E1 and E3 are distinct reactive species in which:

a) R and R' comprise the remainder of the molecules not relevant to this reaction;

b) E1 is attached to R' by a covalent bond which are together designated as E1-R'or R'-E1;

c) E3 is attached to R by a covalent bond which are together designated as E3-R or R-E3;

d) R' represents the part of E1-R not undergoing a chemical reaction;

e) R represents the part of R-E3 not undergoing a chemical reaction;

f) E1 undergoes a chemical reaction with E3 to form the product E1'-E3' and a byproduct F, wherein F is selected from the group consisting of $2H^+$ and $2e^-$, H_20, and any other byproduct;

g) where H^+ is the cation of any isotope of hydrogen and e^- is an electron;

h) where H represents any isotope of hydrogen and O represents any isotope of oxygen;

I) where E1' and E3 are covalently bonded;

j) E1 does not undergo a chemical reaction with another E1;

k) E3 does not undergo a chemical reaction with another E3; and

(1) E1 and E3 are selected from the group consisting of a carboxylate, amino, imidazole, sulfhydryl, aldehyde, ester, and any other reactive species;

wherein [J]p, E2, [I]q and [G]m together comprise a linker moiety, and wherein [G]m, [J]p, and [I]q represent, independently, linker molecules (i) having a molecular weight ranging between about 100 daltons and about 2000 daltons, (ii) having a span ranging from about 20 Å to about 300 Å, and (iii) containing a chain of atoms selected from the group consisting of a combination of C, O, N, S, and Ph atoms, connected by single bonds or by double bonds in a manner that does not violate the laws of chemistry and wherein S represents any isotope of sulfur and Ph represents any isotope of phosphorous; and wherein m, p and q represent, independently, an integer from 1 to 50, inclusive;

and wherein E2 is selected from the group consisting of CX, CH, N, PhYZ, PhU and any other moiety capable of forming covalent bonds with $[J]_p$, $[G]_m$, and $[I]_q$ and wherein:

a) C is any isotope of carbon;

b) x is any isotope of any atom capable of forming a single bond with carbon;

c) H is any isotope of hydrogen;

d) N is any isotope of nitrogen;

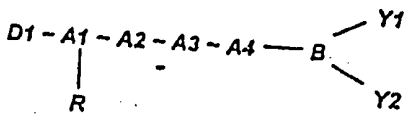
e) Ph is any isotope of phosphorous;

f) Y is any isotope of any atom capable of forming a single bond with phosphorous;

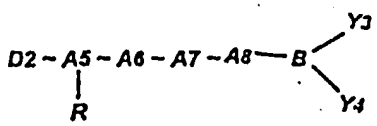
g) Z is any isotope of any atom capable of forming a single bond with phosphorous; and

h) U is any isotope of any atom capable of forming a double bond with phosphorous.

36. The compound of claim 17 wherein the following structures



and



represent, independently, a binding moiety, wherein R represents the remainder of the molecule.

37. The compound of claim 35 wherein (a) [G]m is the side chain of a D- or L- isomer of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine; (b) E2 is D- or L- isomer of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine; (c) E1 and E3 are selected from the group consisting of an amino moiety and a carboxylic acid moiety; and (d) E1 and E3 are distinct from each other.

38. The compound of claim 35 wherein (a) [G]m is the side chain of a D- or L- isomer of lysine, cysteine, glutamic acid, aspartic acid, histidine, arginine, glutamine, and asparagine; (b) E2 is selected from the group consisting of 2-carboxybutyl, 2-carboxypropyl, 2-aminobutyl, 2-aminopropyl, and a hydrocarbon chain with an amino or carboxy side chain; (c) [J]p and [I]q represent, independently, hydrocarbon chains; (d) E1 and E3 are selected from the group consisting of an amino moiety and a carboxylic acid moiety; and (e) E1 and E3 are distinct from each other.

39. A method for stimulating activation of proliferation of human CD26-bearing lymphocytes or CD26-bearing hematopoietic cells, said method comprising contacting said lymphocytes or hematopoietic cells with a proliferation or activation-inducing concentration of the compound of any of claims 1, 17, 35 or 49.

40. The method of claim 39, wherein said contacting is carried out by administering said compound to a human patient suffering from a disease state characterized by inadequate lymphocyte activation or concentration.

41. The method of claim 40, wherein said disease state is caused by HIV infection.

42. The method of claim 40, wherein said compound is administered in conjunction with a second, different agent which stimulates activation or proliferation of said lymphocytes.

43. The method of claim 40, wherein said compound is administered by a route selected from the group consisting of orally, intramuscularly, subcutaneously, and intravenously.

44. The method of claim 39, wherein said contacting of lymphocytes with said compound is carried out *in vitro*.

45. The method of claim 40, wherein said disease state is a neoplasm, and said CD26-bearing lymphocytes are cytolytic or helper T cells.

46. The method of claim 40, wherein said patient is suffering from side effects of chemotherapy or radiation therapy, one of which side effects being a consequence of depletion of cells of the immune system, wherein cells of the immune system are selected from the group consisting of cells derived from lymphoid, erythroid and myeloid lineages.

47. The method of claim 40, wherein said patient suffers from kidney failure resulting in depletion of cells of the immune system.

48. The method of claim 40, wherein said patient suffers from a bone marrow disorder resulting in immunodeficiency.

49. A compound having the formula I:

$$[P^2(R^2)_m]_n - L - P^1R^1$$

wherein P^1 presents a first targeting moiety that mimics the substrate binding site of a protease expressed on the surface of a cell involved in immune system modulation;

R^1 represents a reactive group that reacts with a functional group in the reactive center of the protease;

P^2 represents a second targeting moiety that may be the same or different from the first targeting moiety;

R2 represents a second reactive group that may be the same or different from the first reactive group;

m=0 or 1 and n=a whole number from 1 to 10, and L represents a linker molecule.

50. The compound of claim 49, wherein P^2=P^1 and R^2 is absent or is different from R^1.

51. The compound of claim 49, wherein P^1 selectively binds to a DP IV on a first cell and P^2 selectively binds to a major histocompatibility molecule on an antigen presenting cell.

52. A vaccine comprising the compound of claim 49.

53. A pharmaceutical composition comprising the compound of claims 1, 17, 35 or 49, in a pharmaceutically acceptable carrier.

54. A method for manufacturing a pharmaceutical composition comprising:

placing the compound of claims 1, 17, 35, or 49 in a pharmaceutically acceptable carrier.

55. The method of claim 39, wherein administering comprises obtaining the T cells, bone marrow cells, stem cells or early lineage progenitor cells from the subject, contacting the isolated cells with the compound *ex vivo* in an amount effective to stimulate the cells, and reintroducing the cells to the subject.

56. A method for treating an autoimmune condition comprising:

administering the compound of claims 1, 17, 35, or 49 to a subject in need of such treatment in an amount effective to inhibit the autoimmune condition in the subject.

57. The method of claim 40, wherein said patient suffers from immunodeficiency symptoms resulting from depletion of cells of the immune system.

58. The compound of claim 49, wherein P^2 is a retroinverso peptide.

59. The compound of claim 49, wherein P^2 is a tumor antigenic peptide.

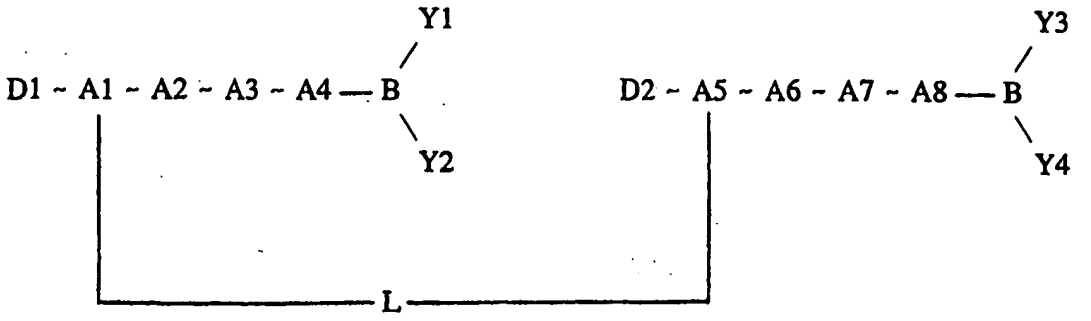


FIG. 1A

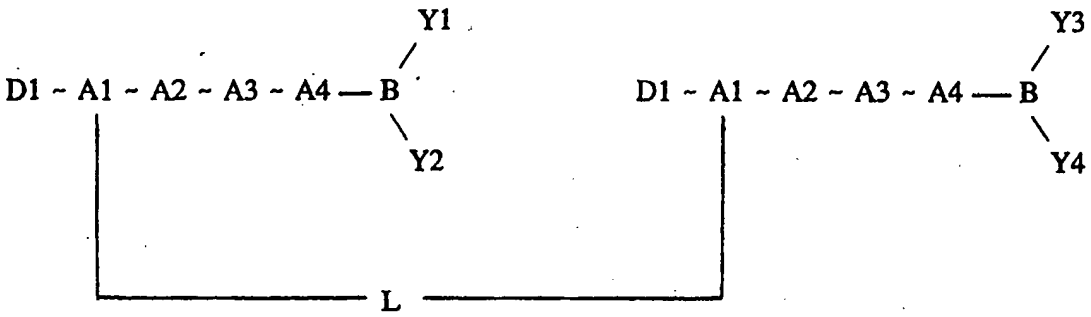


FIG. 1B

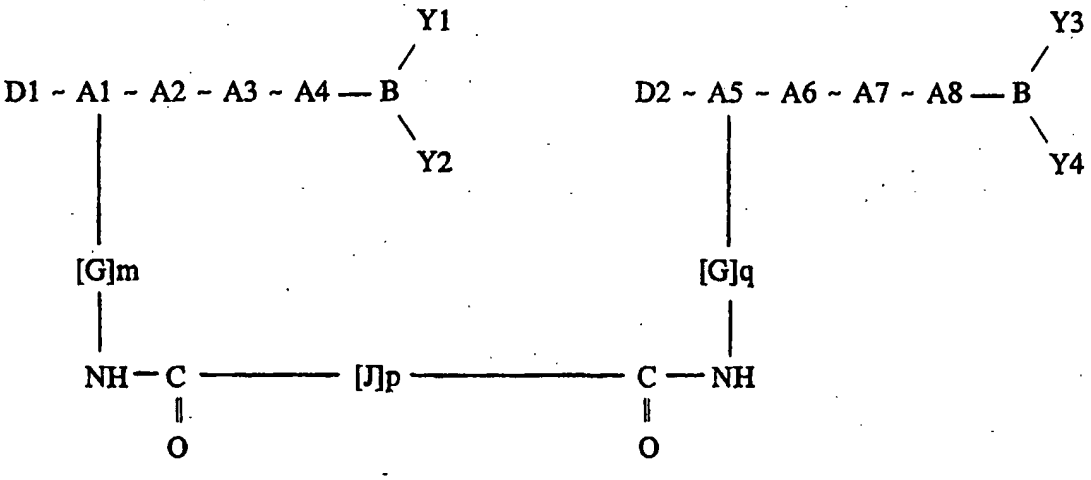


FIG. 1C

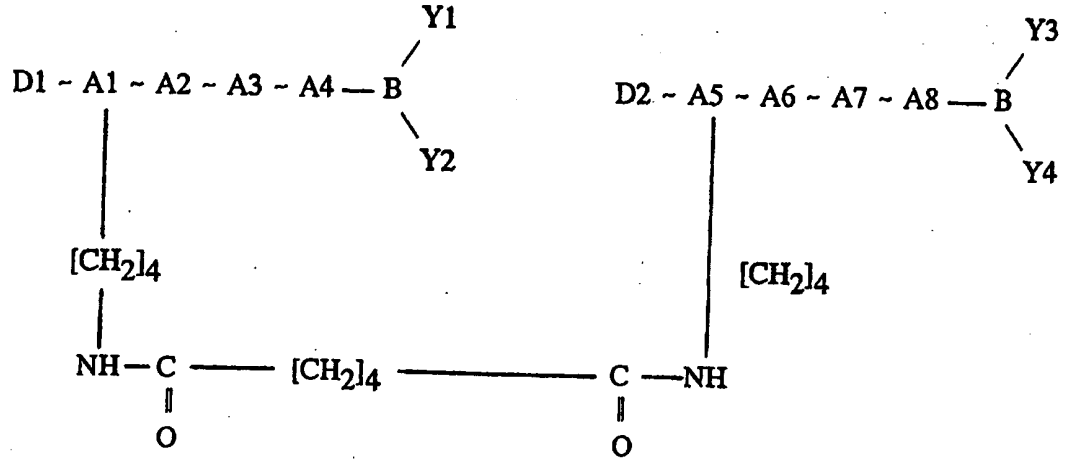

Y1
D1 ~ A1 ~ A2 ~ A3 ~ A4 — B
Y2
Y3
D2 ~ A5 ~ A6 ~ A7 ~ A8 — B
Y4
[CH2]4
[CH2]4
NH—C
[CH2]4
C —NH
O
O

FIG. 1D

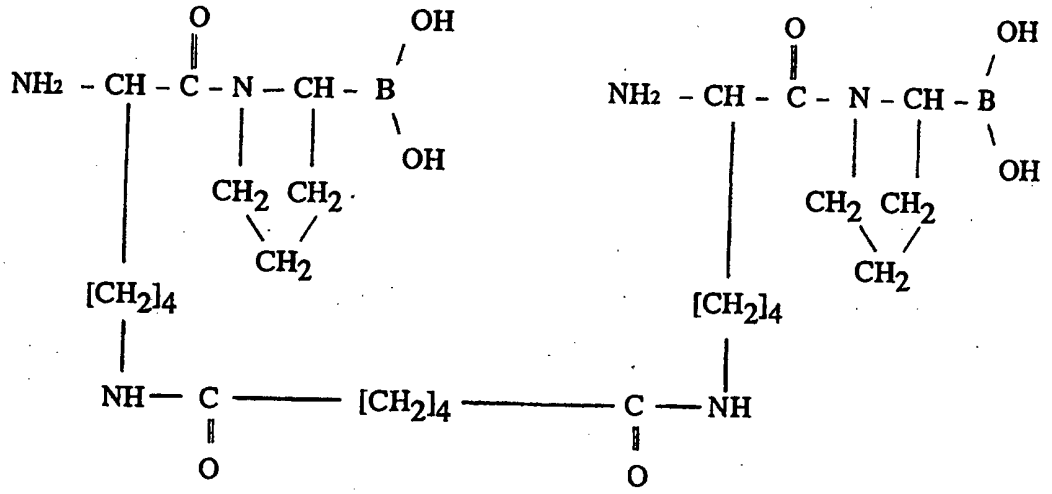

O
OH
NH2 - CH - C - N — CH - B
OH
CH2 CH2
CH2
[CH2]4
NH— C
[CH2]4
C — NH
O
O
O
OH
NH2 - CH - C - N - CH - B
OH
CH2 CH2
CH2
[CH2]4

FIG. 1E

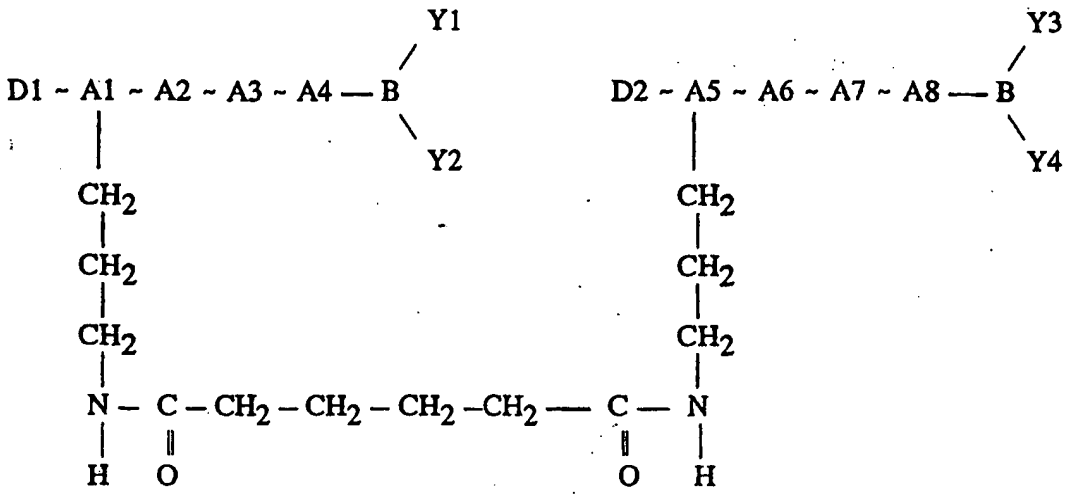

Y1
D1 ~ A1 ~ A2 ~ A3 ~ A4 — B
Y2
Y3
D2 ~ A5 ~ A6 ~ A7 ~ A8 — B
Y4
CH2
CH2
CH2
N — C — CH2 — CH2 — CH2 — CH2 — C — N
H O O H

FIG. 1F

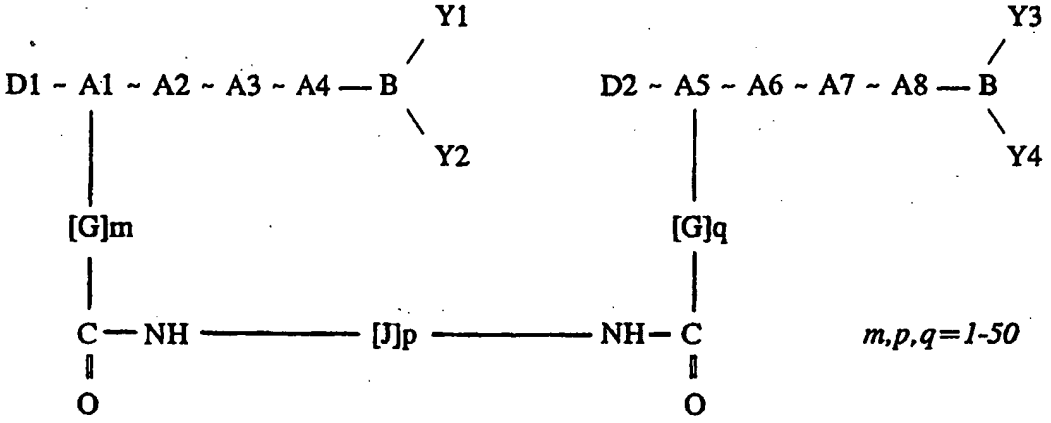

Y1
D1 ~ A1 ~ A2 ~ A3 ~ A4 — B
Y2
Y3
D2 ~ A5 ~ A6 ~ A7 ~ A8 — B
Y4
[G]m
[G]q
C — NH — [J]p — NH — C
O O
m,p,q=1-50

FIG. 1G

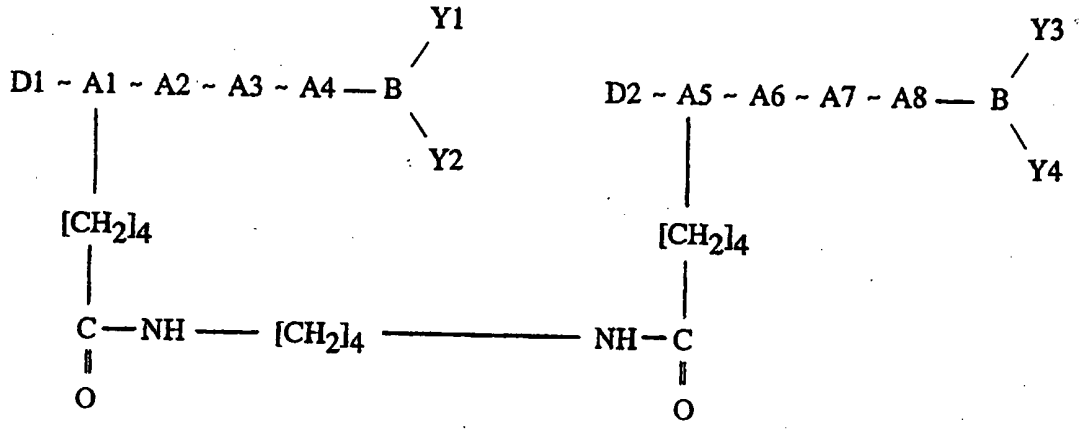

D1 ~ A1 ~ A2 ~ A3 ~ A4 — B
Y1
Y2
D2 ~ A5 ~ A6 ~ A7 ~ A8 — B
Y3
Y4
[CH2]4
C — NH — [CH2]4 — NH — C
O

FIG. 1H

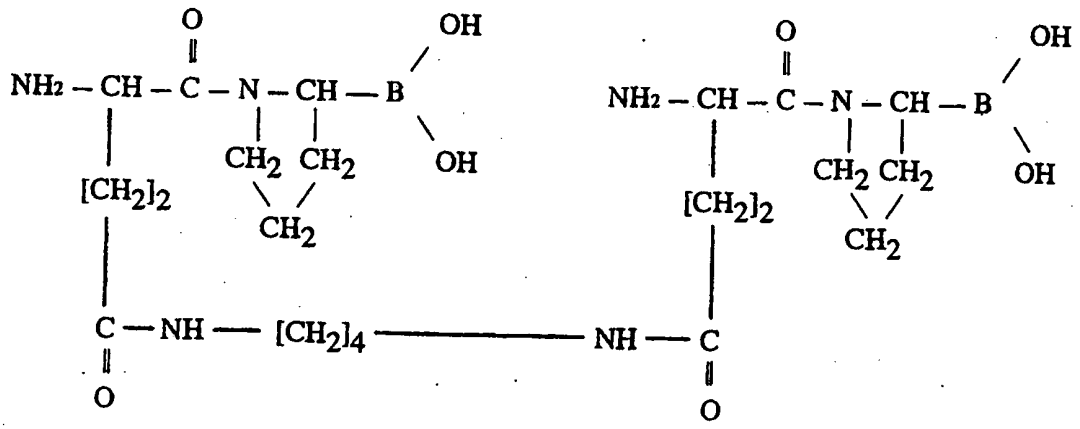

NH2 — CH — C — N — CH — B
OH
OH
[CH2]2
CH2 CH2
CH2
C — NH — [CH2]4 — NH — C
O

FIG. 1I

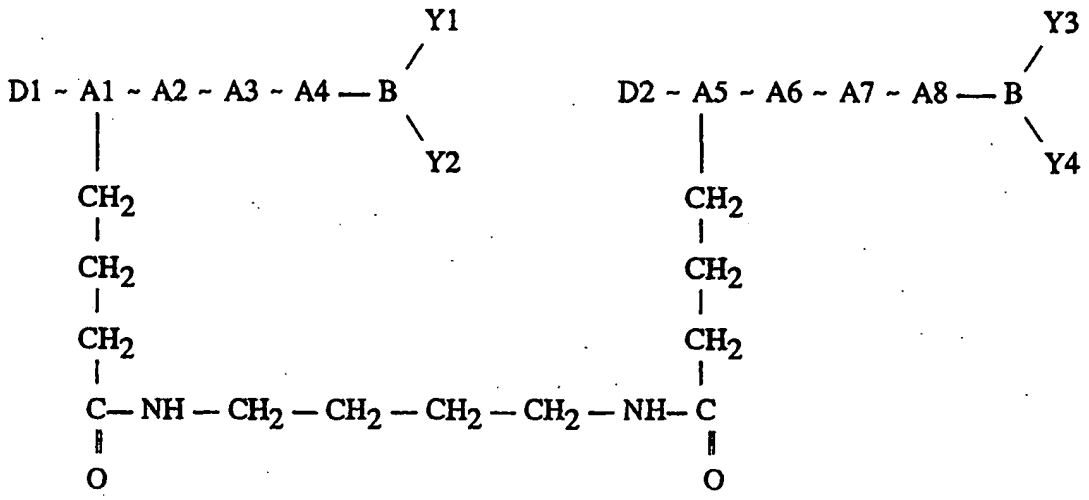
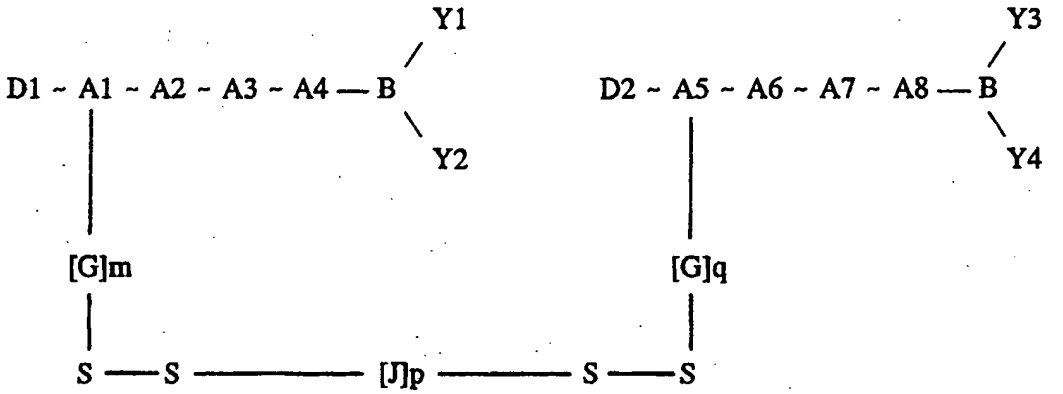


FIG. 1J



m,p,q=1-50

FIG. 1K

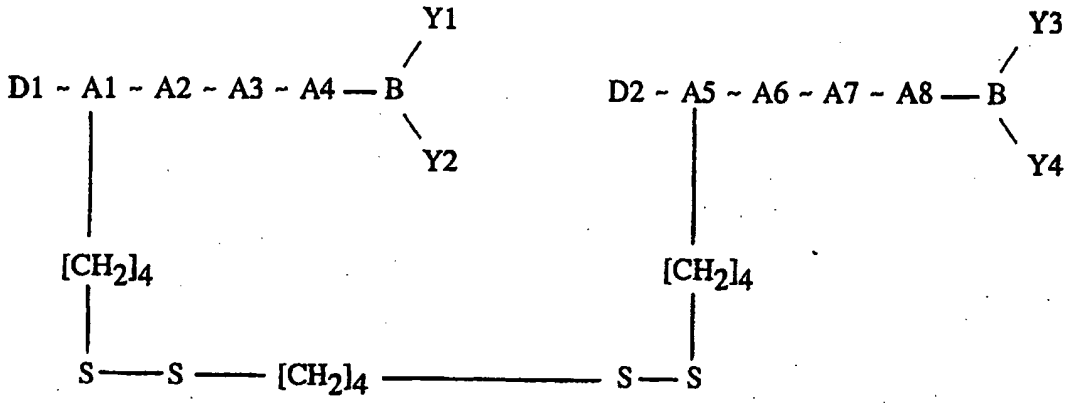

Y1
D1 ~ A1 ~ A2 ~ A3 ~ A4 — B
Y2
Y3
D2 ~ A5 ~ A6 ~ A7 ~ A8 — B
Y4
[CH2]4
[CH2]4
S — S — [CH2]4 — S — S

FIG. 1L

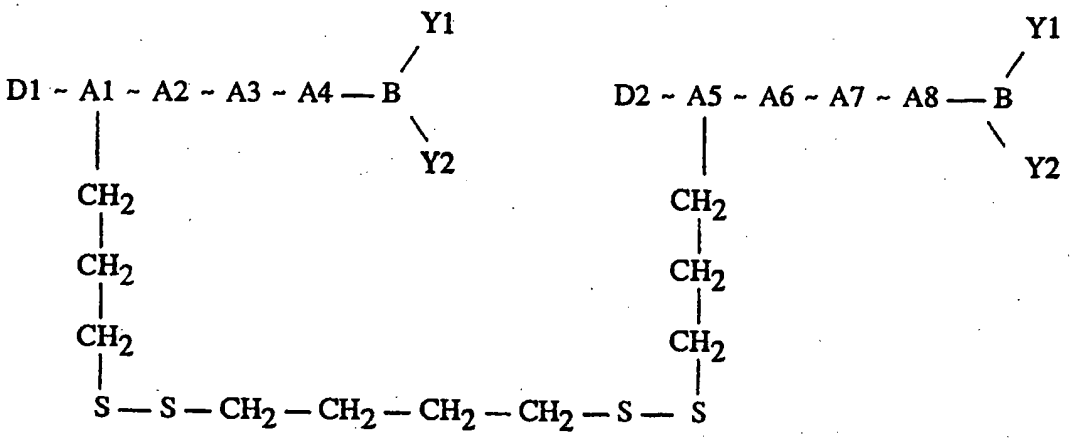

Y1
D1 ~ A1 ~ A2 ~ A3 ~ A4 — B
Y2
Y1
D2 ~ A5 ~ A6 ~ A7 ~ A8 — B
Y2
CH2
CH2
CH2
CH2
CH2
CH2
S — S — CH2 — CH2 — CH2 — CH2 — S — S

FIG. 1M

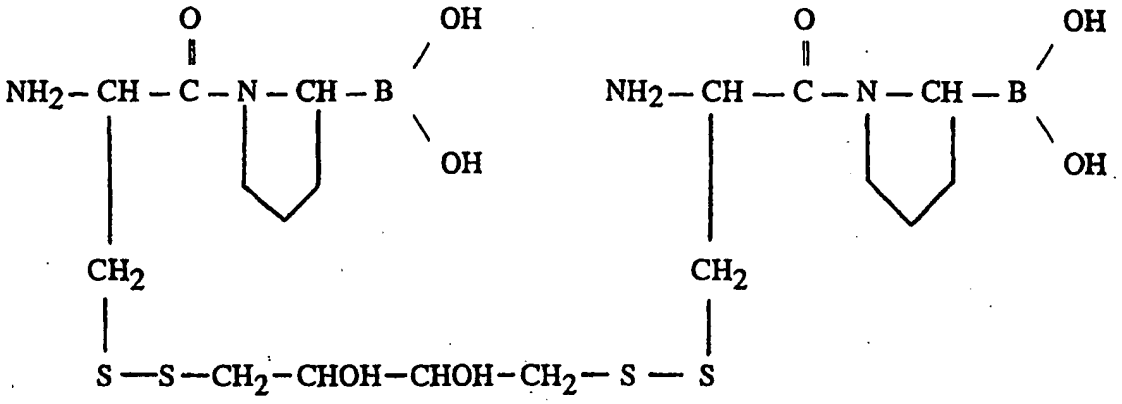


FIG. 1N

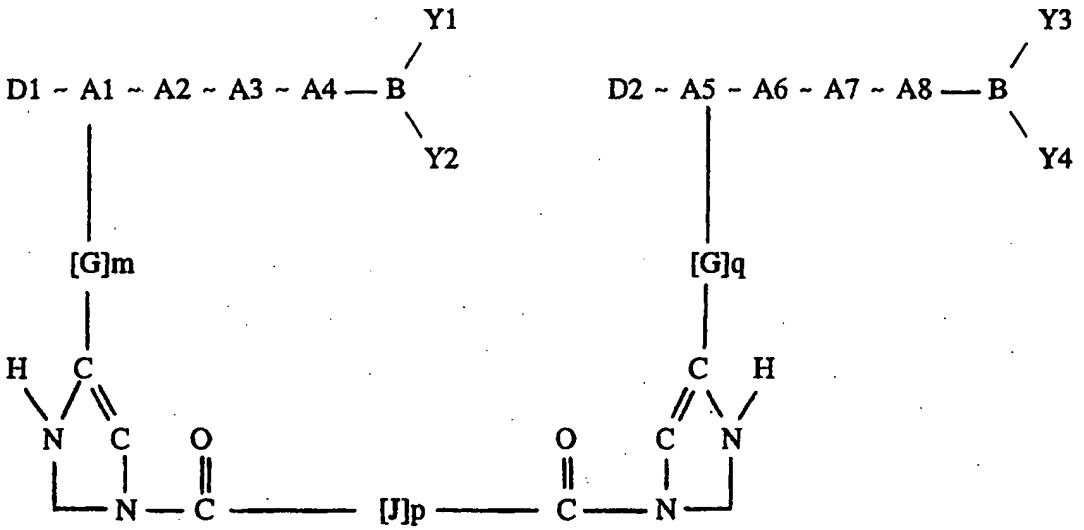


FIG. 1O

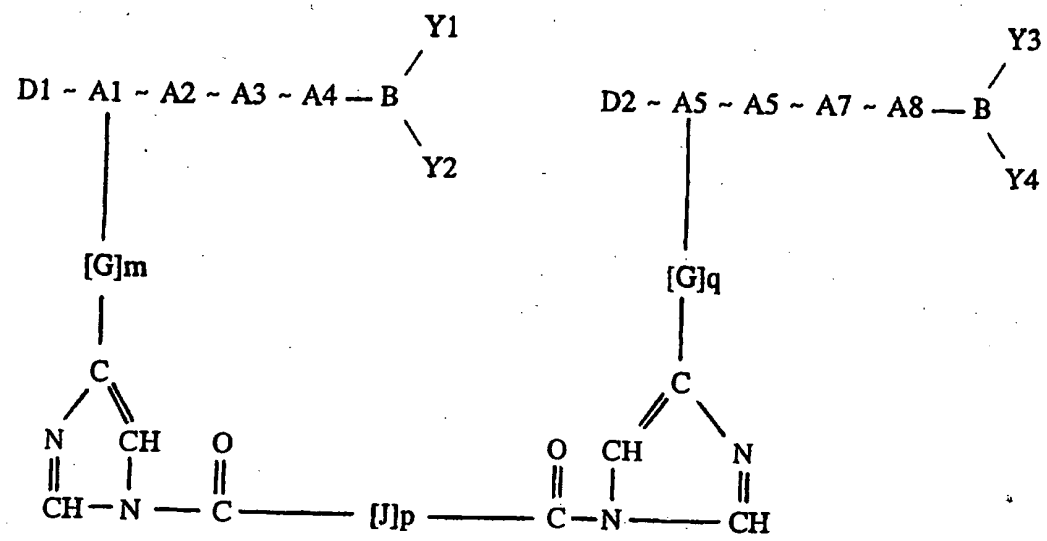

D1 ~ A1 ~ A2 ~ A3 ~ A4 — B
Y1
Y2
D2 ~ A5 ~ A5 ~ A7 ~ A8 — B
Y3
Y4
[G]m
[G]q
[J]p

FIG. 1P

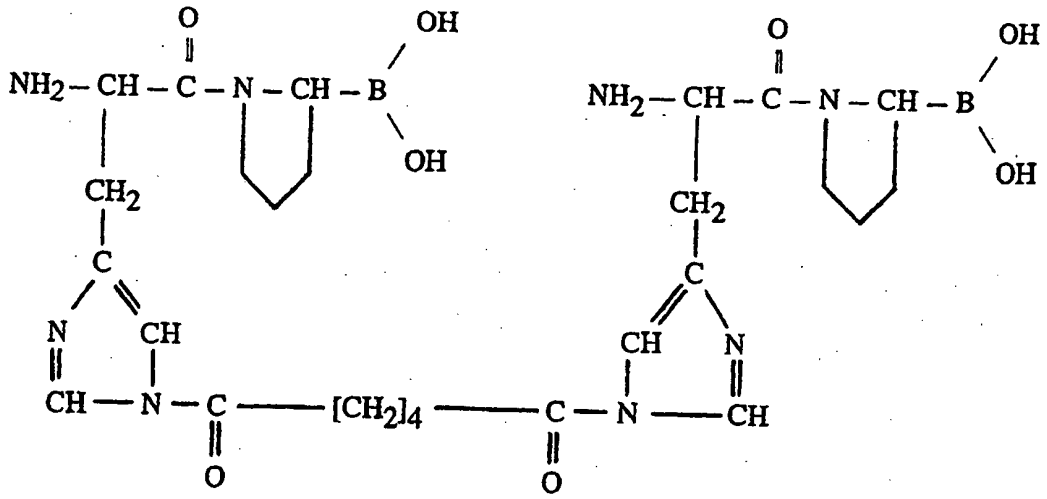
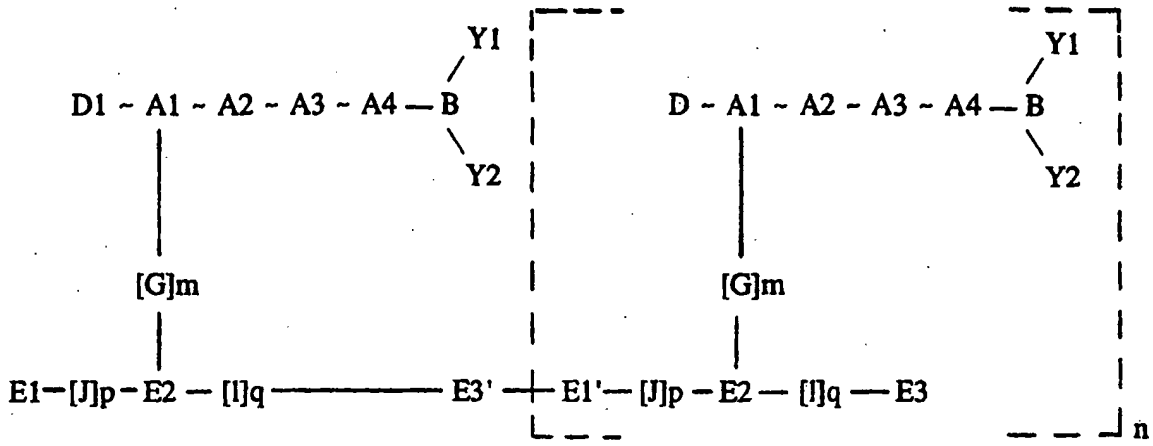

NH2 — CH — C — N — CH — B
OH
OH
CH2
[CH2]4

FIG. 1Q



R - E3 + E1 - R' → R - E3' - E1' - R' + F
F ≡ $2H^+ + 2e^-$, H_2O, or other byproduct
R & R' ≡ remainder of molecules not relevant to the reaction

FIG. 1R

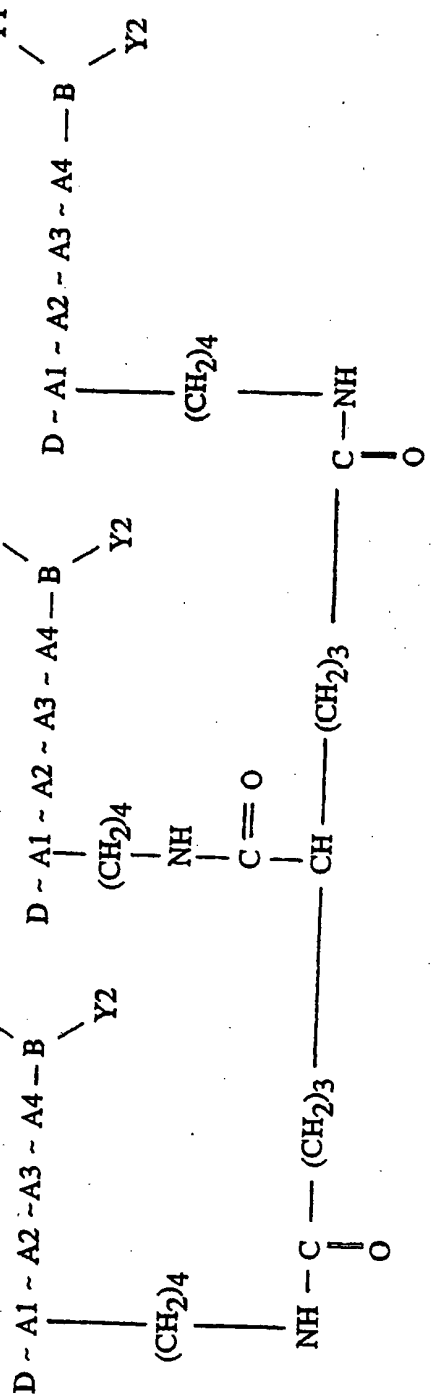


FIG. 15

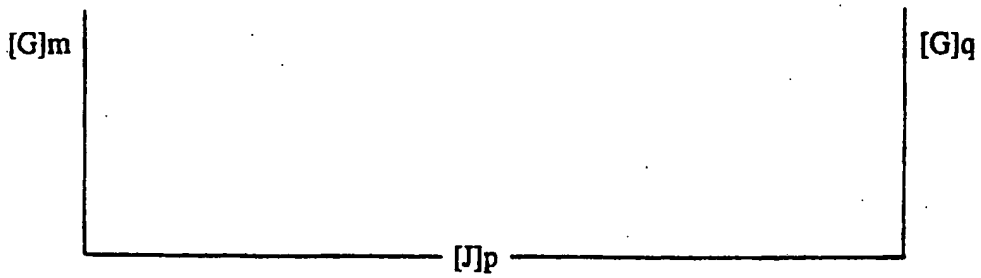


FIG. IT

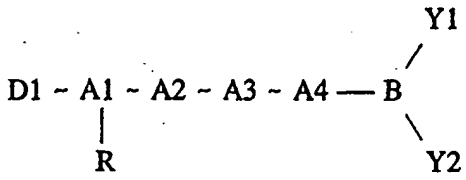


FIG. IU

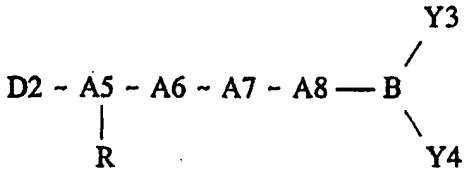


FIG. IV

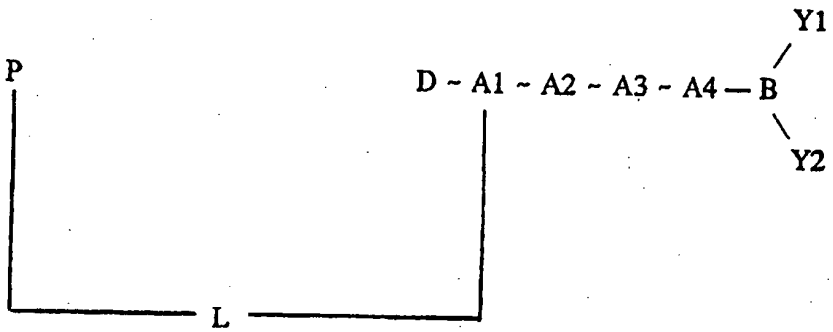


FIG. 2A

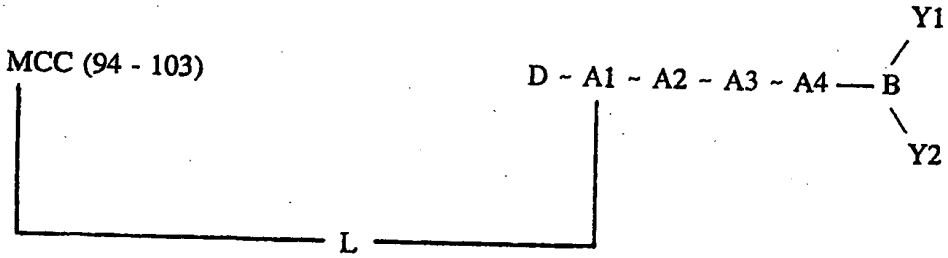


FIG. 2B

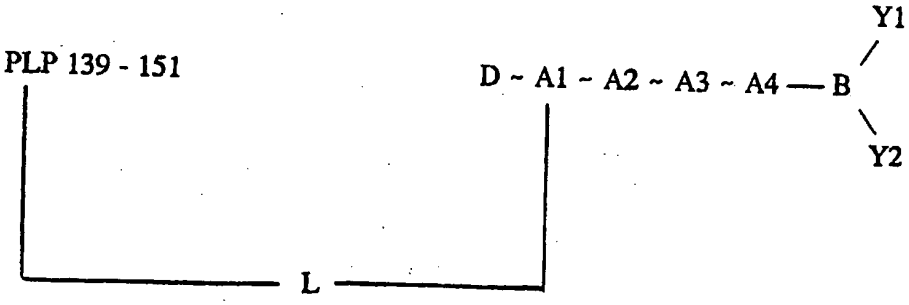


FIG. 2C

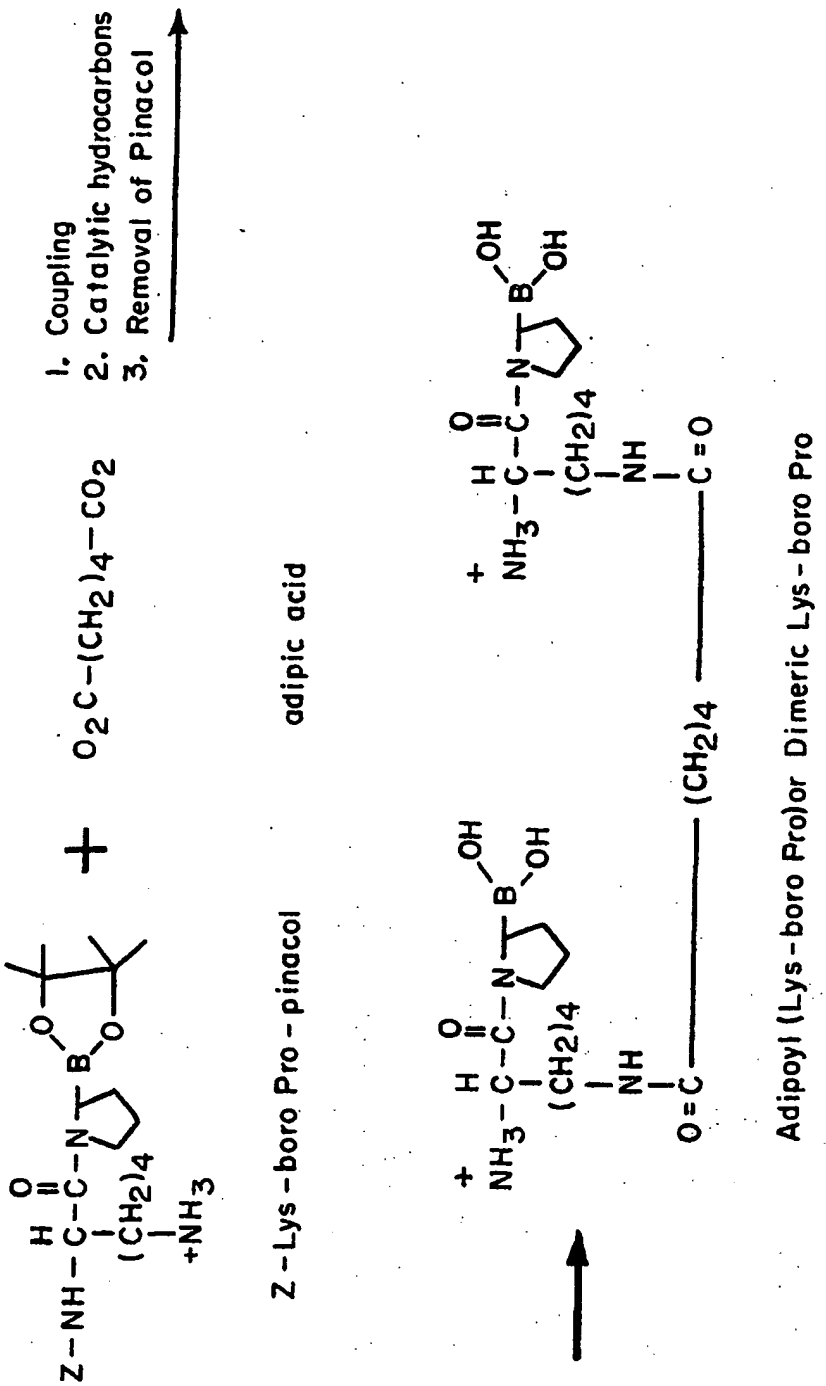

1. Coupling
2. Catalytic hydrocarbons
3. Removal of Pinacol
Z-Lys-boro Pro-pinacol
adipic acid
Adipoyl (Lys-boro Pro)or Dimeric Lys-boro Pro

FIG. 3

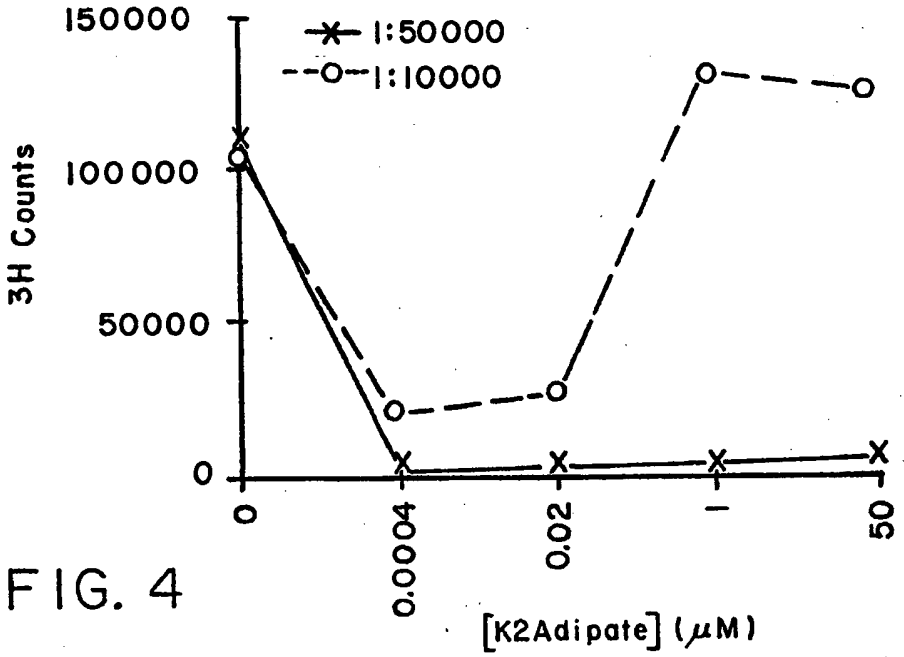

150000
100000
50000
0
3H Counts
1:50000
1:10000
0
0.0004
0.02
1
50
[K2Adipate] (μM)

FIG. 4

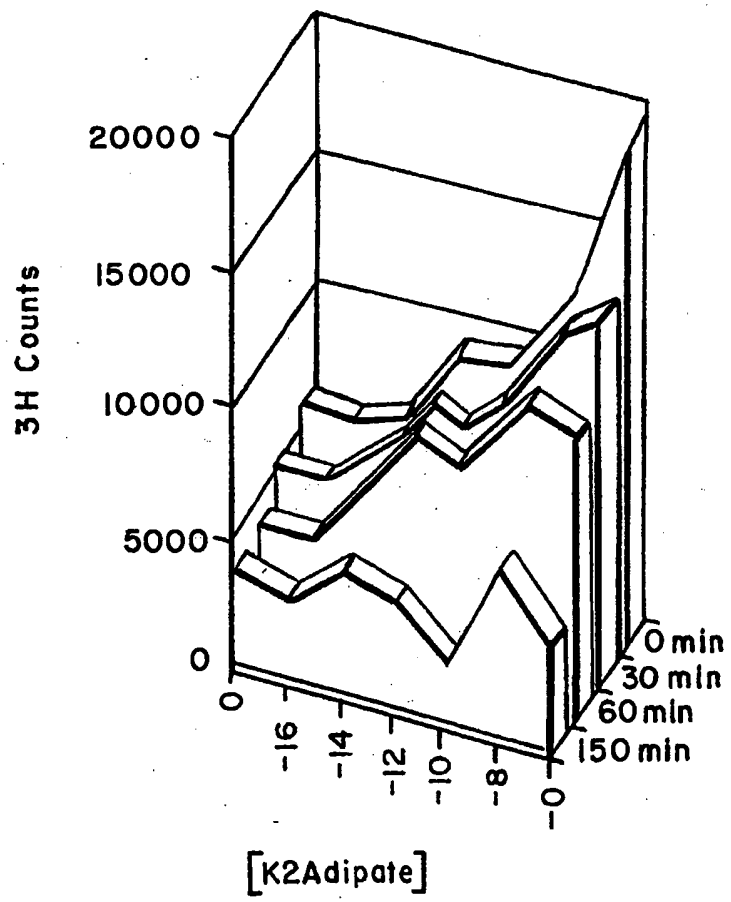

20000
15000
10000
5000
0
3H Counts
0
-16
-14
-12
-10
-8
-0
[K2Adipate]
0 min
30 min
60 min
150 min

FIG. 5

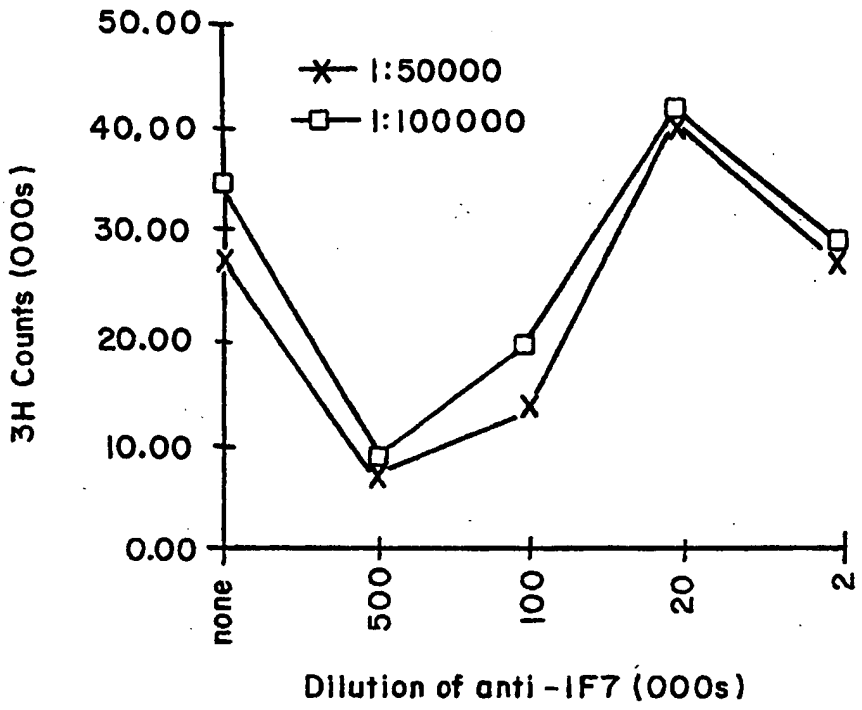

50.00
40.00
30.00
20.00
10.00
0.00
1:50000
1:100000
3H Counts (000s)
none
500
100
20
2
Dilution of anti-IF7 (000s)

FIG. 6

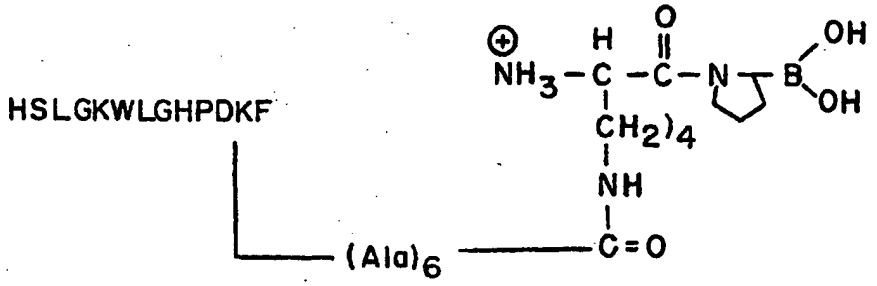

HSLGKWLGHPDKF
(Ala)6
NH3
H
C
O
C
N
B
OH
OH
CH2)4
NH
C=O

FIG. 8

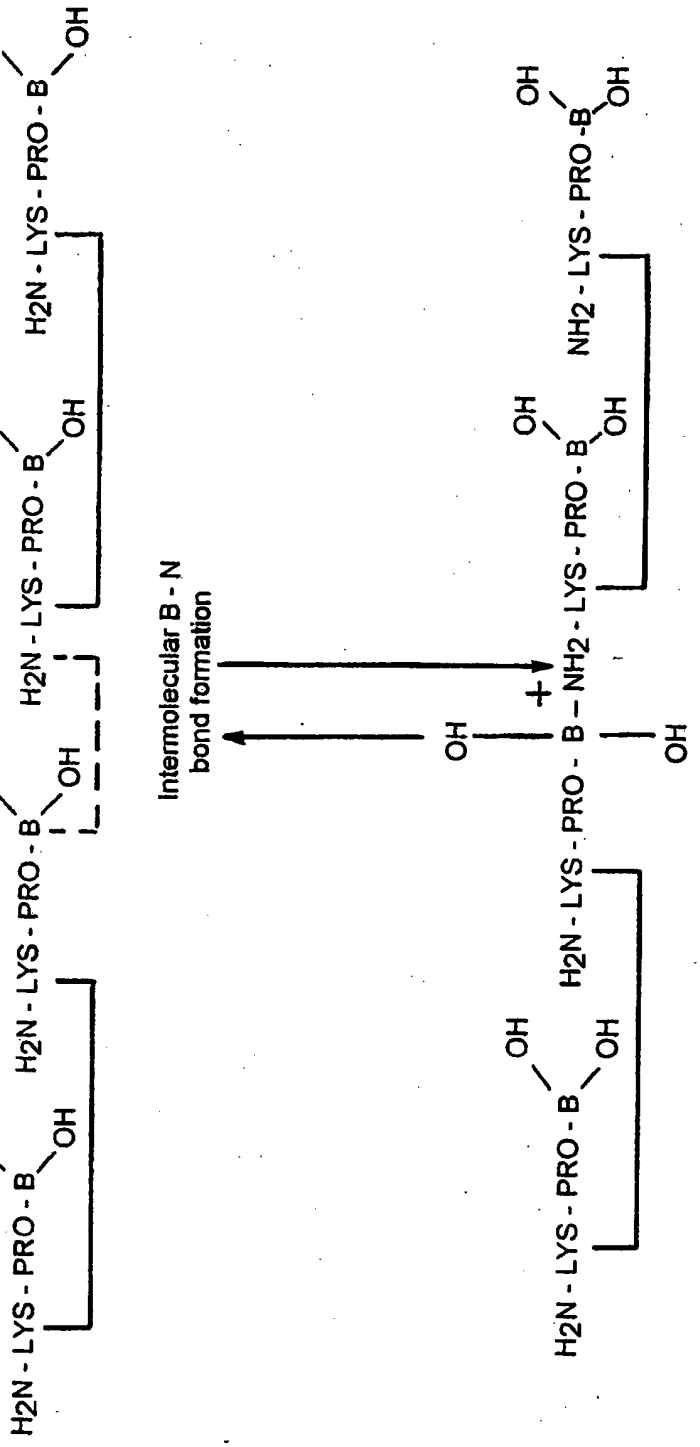

Intermolecular B - N
bond formation
H2N - LYS - PRO - B
OH
OH
H2N - LYS - PRO - B
OH
OH
H2N - LYS - PRO - B
OH
OH
H2N - LYS - PRO - B
OH
OH
+
NH2 - LYS - PRO - B
OH
OH
NH2 - LYS - PRO - B
OH
OH
H2N - LYS - PRO - B
OH
OH
H2N - LYS - PRO - B
OH
OH

FIG. 7

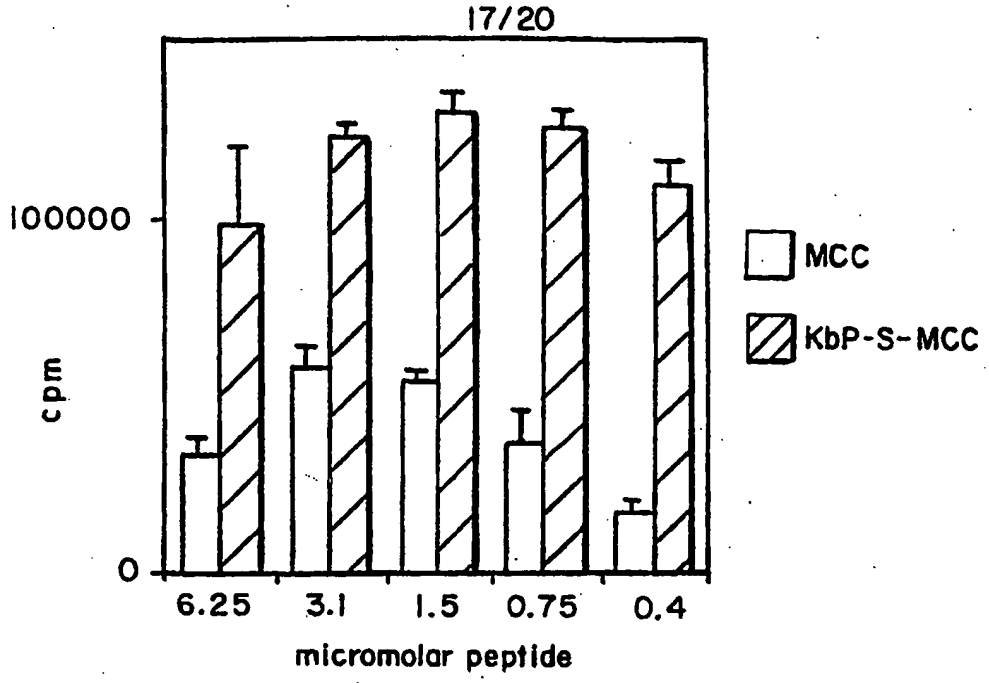

100000
0
cpm
6.25
3.1
1.5
0.75
0.4
micromolar peptide
MCC
KbP-S-MCC

FIG. 9

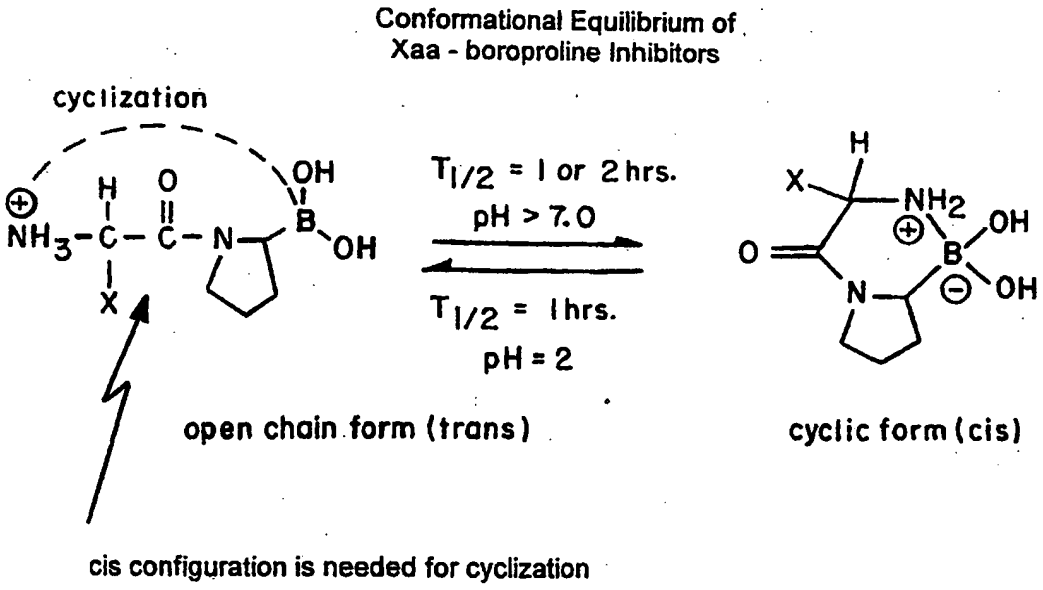

Conformational Equilibrium of
Xaa - boroproline Inhibitors
cyclization
$T_{1/2}$ = 1 or 2 hrs.
pH > 7.0
$T_{1/2}$ = 1 hrs.
pH = 2
open chain form (trans)
cyclic form (cis)
cis configuration is needed for cyclization

FIG. 10

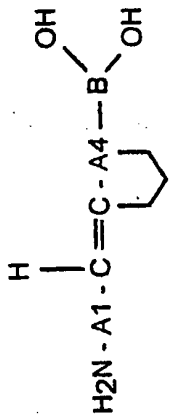


FIG. IIA

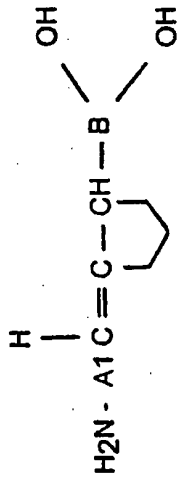


FIG. IIB

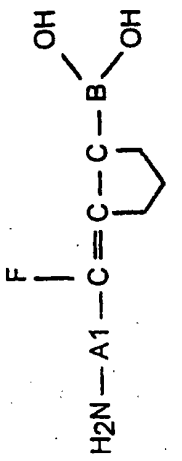
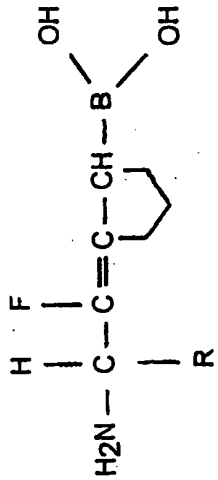


FIG. IIC



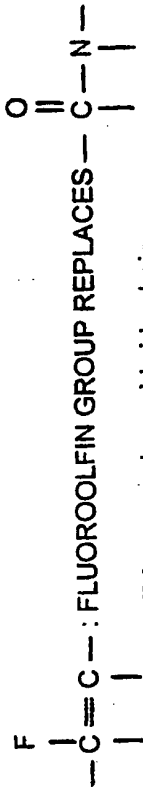


FIG. IID

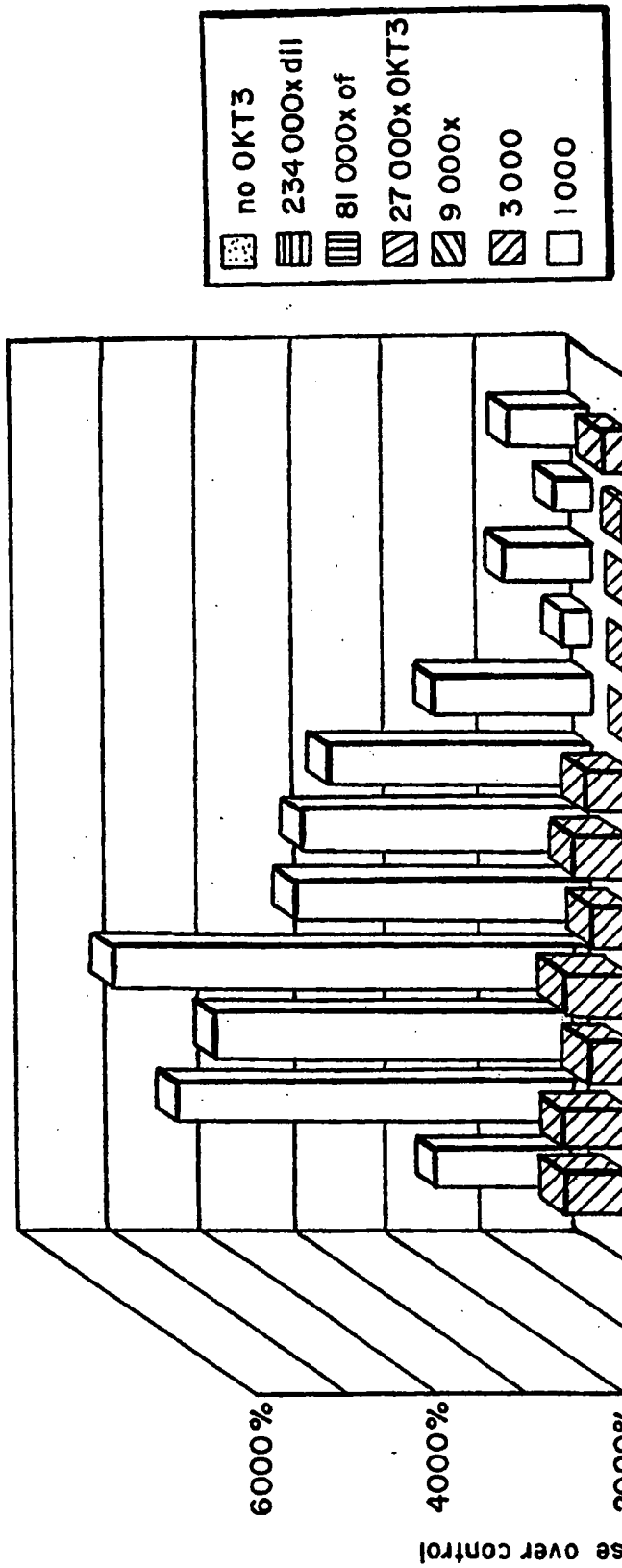

no OKT3
234000x dll
81000x of
27000x OKT3
9000x
3000
1000
Increase over control
6000%
4000%
2000%
0%
-5 -6 -7 -8 -9 -10 -11 -12 -13 -14 -15 none
(KbP)2 EGS (log10M)

FIG. 12

(KbP)$_2$ EGS

EGS: Ethylene glycolbis (succinimidylsuccinate)

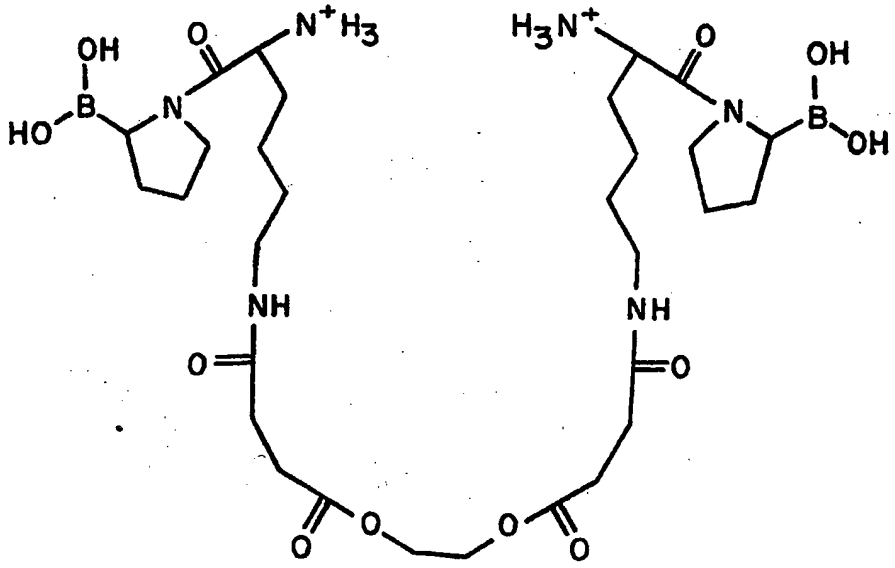


FIG. 13

CORRECTED VERSION*

PCT

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(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: BACHOVCHIN, William, W. [US/US]; 71 Warwick Road, Melrose, MA 02176 (US).

(74) Agent: PLUMER, Elizabeth, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(54) Title: MULTIVALENT COMPOUNDS FOR CROSS-LINKING RECEPTORS AND USES THEREOF

(57) Abstract

Synthetic cross-linking homobivalent and heterobivalent compounds have been designed and developed. These compounds are low in molecular weight, have antagonistic or agonistic activity, and induce the association between two identical or similar natural receptors (homobivalent compounds) or induce the association between two different natural receptors (heterobivalent compounds).

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BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/US 97/11279

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 C07K14/435 C07K14/16 C07K14/535 C07F5/02
A61K38/16 A61K31/69

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C07F A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 15309 A (FERRING BV ;JENKINS PAUL D (GB); JONES D MICHAEL (GB); SZELKE MICH) 8 June 1995 see page 1, paragraph 4 - page 3, paragraph 10 see page 4, paragraph 4; claims; examples; table 7 ---	1-16, 35-59
A	WO 95 29190 A (PASTEUR INSTITUT) 2 November 1995 see page 26, paragraph 4 - page 27, paragraph 1; claims; examples ---	1-16, 35-59
A	US 5 329 028 A (ASHKENAZI AVI J ET AL) 12 July 1994 see column 2, line 63 - column 3, line 23; claims; examples --- -/--	1,39-41

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
7 January 1998	21.01.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ...nal Application No
PCT/US 97/11279

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. Wijdenes et al. 'Monoclonal Antibodies (mAb) against GP130 imitating Cytokines which use the gp130 for Signal Transduction' in: The 9th International Congress of Immunology, Abstract Book, 23-29 July, 1995, San Francisco, CA, USA XP002051385 abstract no. 1794 on page 303 see abstract ---	1

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int. .ational application No.
PCT/US 97/11279

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 97/11279

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 1-7, 9-10, 16-23, 25-26, 29-31, 35-59

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

In view of the extremely large number of compounds falling under claims 1-7, 9-10, 16-23, 25-26, 29-31, 35-38, 49-51, 58-59 and claims 39-48, 52-57 referring to them and of the absence of any technical support for these compounds in the description, the ISA considers that it is not economically reasonable to draw a search report covering the entire subject matter characterized above.
The search has therefore been limited to the real examples given in the description and closely related ones and includes compounds having the alleged activities.

Remark : As far as claims 39-43, 45-48 and 55-57 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 97/11279

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9515309 A	08-06-95	AU 1113395 A	19-06-95
		CA 2178066 A	08-06-95
		CN 1141033 A	22-01-97
		CZ 9601595 A	15-01-97
		EP 0731789 A	18-09-96
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		HU 76274 A	28-07-97
		JP 9509921 T	07-10-97
		NO 962269 A	30-07-96
		PL 314838 A	30-09-96
		ZA 9409525 A	02-08-95
WO 9529190 A	02-11-95	FR 2719049 A	27-10-95
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		EP 0756603 A	05-02-97
US 5329028 A	12-07-94	US 5605791 A	25-02-97